Electronic Supplementary Material (ESI) for Analyst

Supporting Information for

Development of a pH-activatable fluorescent probe and its application for visualizing cellular pH change

Rong Huang, [a‡] Shengyong Yan, [a‡] Xiaolong Zheng, [a‡] Fengling Luo, [c] Minggang Deng, [a] Boshi Fu, [a] Yijie Xiao, [a] Xi Zhao, [a] Xiang Zhou [ab]*

College of Chemistry and Molecular Sciences, Key Laboratory of Biomedical Polymers of Ministry of Education, State Key Laboratory of Virology, Wuhan University, Hubei, Wuhan, 430072, P. R. of China, Corresponding Author Email Address: xzhou@whu.edu.cn

Table of Contents

Apparatus

Fluorescent emission spectra were collected from 500-700 nm on PerkinElmer LS 55 with an excitation wavelength of 468 nm, the excitation and emission slit widths were both 10 nm. Quartz cuvettes with 2 mL volume used for emission measurements. UV-Vis absorption spectra were collected on SHIMADZU UV-2550 from 200-700nm with 600 μL quartz cuvettes. Unless otherwise specified, all spectra were taken at 25 ℃ in 10mM potassium phosphate buffers. All pH measurements were performed with a pB-10 pH-meter (Sartorius, Shanghai, China) with a combined glass-calomel electrode. ^{1}H and ^{13}C NMR spectra were recorded on Varian Mercury 300 spectrometers, respectively. HRMS were recorded on a Brucker APEX IV (7.0 T). Florescent images were acquired on Nikon Confocal Laser Scanning Microscope (TE2000, Japan) with an oil objective lens (×60). Flow cytometry data were recorded by Beckmen Coulter Eltra flow cytometer Epics Altra II (Beckman, USA).

Materials

All solvents and reagents were commercially available and used without further purification unless for special needs. HeLa cells were purchased from China Center for Type Culture Collection. LysoTracker Red was purchased from beyotime, China.

Optical Properties and Quantum Efficiency of Fluorescence

Optical properties of compounds were examined in 10mM potassium phosphate buffer (pH=2, 10).The constants pKa of the four compounds were determined in analysis of fluorescence intensity changes as a function of pH by using Henderson-Hasselbalch equation:

$$pKa = pH - \log\frac{FImas - FI}{FI - FImin}$$

where FI is the observed fluorescence intensity at a fixed wavelength 533nm, FImax and FImin are the corresponding maximum and minimum respectively.

For determination of the quantum efficiency of fluorescence (Yu), Rhodamine 6G in water (Ys = 0.95) was used as a standard. Values were calculated according to the following equation.

$$Yu = Ys \times \frac{Fu}{Fs} \times \frac{As}{Au}$$

Where s means standard, u means sample, A means absorbance at the excitation wavelength, F means integrate area under the fluorescence spectra on an energy scale.

.

Cell Culture

HeLa human cervical carcinoma cells (CCTCC, China) were cultured in DMEM (Hyclone, China) supplemented with 10% FBS (Hyclone), penicillin (100 units/ml), and streptomycin (100 ug/ml). All the cells were maintained in a humidified atmosphere of 5/95 (v/v) of CO_2/air at 37℃.

Confocal Imaging of colocalization

10^5 Cells seeded on 35-mm glass-bottomed dishes (Nest) were washed with 1 mL PBS, then

incubated in DMEM containing 10% FBS, 5 μM FoPz and 5 nM Lysotracker Red for 1 h at 37℃, washed with 1mL PBS twice, and mounted on the microscope stage. The light source was a white-light laser. The excitation wavelength was 488 and 543 nm, and the emission wavelength was 500-530 nm (FoPz), or 600-630 nm (Lysotracker Red), respectively.

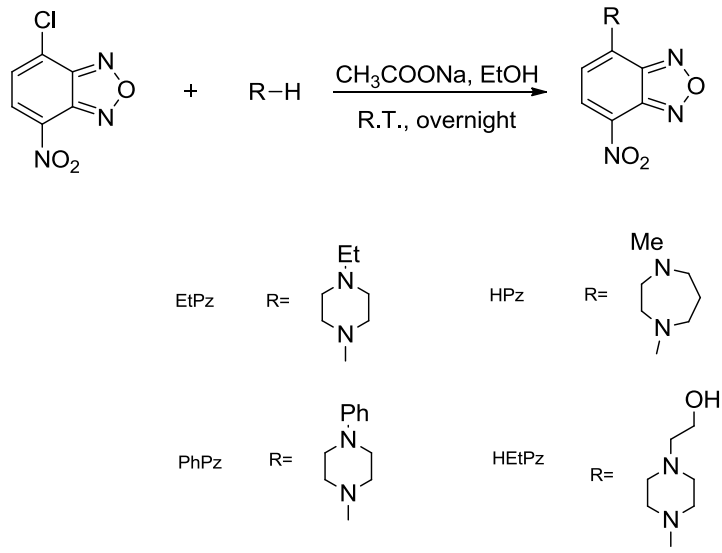
Confocal Imaging of changing pH

Cells seeded on 35-mm glass-bottomed dishes (Nest) were washed with 1 mL PBS, then incubated in DMEM containing 10% FBS, 5 μM FoPz for 1 h at 37℃, washed with phosphate buffers at pH 6, 7 and 8 for two times, respectively. The excitation wavelength was 488 and the emission wavelength was 500-530 nm.

Flow Cytometry Assay

Generally, probe FoPz (5 μM) was incubated with 2×10^5 cells at 37℃ for 1h. After digestion, cells were immediately immersed in various pH buffers for 5 min and determined with flow cytometer by counting 10,000 events. Flow cytometer at high rate by counting the FoPz-labeled events appearing in the upright (UR) region for 2 min.

General procedure for the synthesis of 7-nitro-2,1,3-benzoxadiazole derivates (NBD)



4-(4-Ethylpiperazin-1-yl)-7-nitro-2,1,3-benzoxadiazole (EtPz)

Sodium acetate (123 mg, 1.5 mmol) was added to a solution of 4-chloro-7-nitrobenzo -2-oxa-1,3-diazole (200 mg, 1 mmol) and 1-ethylpiperazine (171 mg, 1.5 mmol) in ethanol (3 mL) in small portions. The reaction mixture was stirred at room temperature over night, then, the solution was filtered and washed with ethanol to afford the product as a red solid (185 mg, 0.67 mmol). Yield 67%.

^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) 8.42 (d, 1 H, J = 8.1 Hz), 6.31 (d, 1 H, J = 8.1 Hz), 4.15 (s, 4 H), 2.71 (s, 4 H), 2.51 (q, 2 H, J = 7 Hz) , 1.16 (t, 3 H, J =6.9 Hz) ; ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 145.4, 145.0, 138.3, 135.4, 123.5, 102.7, 52.5, 52.3, 49.7, 12.2; HRMS (ESI) calcd for

$C_{12}H_{16}N_5O_3[M+H]^+$: 278.1253; found: 278.1245.

4-Nitro-7-(4-phenylpiperazin-1-yl)-2,1,3-benzoxadiazole (PhPz)

The preparative procedure was the same as that for PhPz except that 1-phenylpiperazine was the amine used. Yield 65%.

^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) 8.48 (d, 1 H, *J* = 8.4 Hz), 7.33 (t, 2 H, *J* = 8.4 Hz), 6.97 (m, 3 H), 6.37 (d, 1 H, *J* = 9 Hz), 4.29 (s, 4 H), 3.49 (t, 4 H, *J* = 4.8 Hz); ^{13}C NMR (DMSO, 75 MHz) δ: 151.4, 146.7, 146.2, 137.7, 130.4, 122.0, 120.4, 116.4, 104.8, 50.4, 48.8. HRMS (ESI) calcd for $C_{16}H_{15}N_5O_3Na[M+Na]^+$:348.1073; found: 348.1062.

4-(4-Methyl-1,4-diazepan-1-yl)-7-nitro-2,1,3-benzoxadiazole (HPz)

The preparative procedure was the same as that for HPz except that 1-methyl-1,4-diazepane was the amine used. Yield 55%.

^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) 8.38 (d, 1 H, *J* = 8.7 Hz), 6.16 (d, 1 H, *J* = 8.7 Hz), 4.30 (br, 2 H), 4.04 (br, 2 H), 2.90 (s, 2 H), 2.68 (s, 2 H), 2.43 (s, 3 H), 2.18 (s, 2 H) ; ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 145.5, 144.7, 144.5, 135.4, 122.0, 101.0, 56.8, 52.2, 52.0, 46.7, 26.8; HRMS (ESI) calcd for $C_{12}H_{16}N_5O_3[M+H]^+$: 278.1253; found: 278.1243.

2-(4-(7- nitro-2,1,3-benzoxadiazole-4-yl)piperazin-1-yl)ethanol (HEtPz)

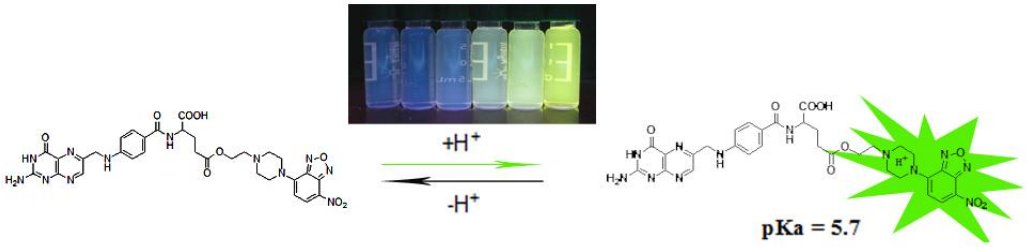
The preparative procedure was the same as that for HEtPz except that 2-(piperazin-1-yl)ethanol was the amine used.

H NMR ($CDCl_3$, 300 MHz) δ (ppm) 8.44 (d, 1 H, *J* = 8.4 Hz), 6.33 (d, 1 H, *J* = 8.4 Hz), 4.13 (s, 4 H), 3.73 (s, 2 H), 2.80 (s, 4 H), 2.67 (s, 2 H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 149.6, 149.0, 140.6, 125.3, 107.9, 64.0, 62.9, 57.1, 53.8; HRMS (ESI) calcd for $C_{12}H_{16}N_5O_4[M+H]^+$: 294.1202; found: 294.1199.

2-(4-((2-amino-4-oxo-3,4-dihydropteridin-6-yl)methylamino)benzamido)-5-(2-(4-(7-nitroben zo[c][1,2,5]oxadiazol-4-yl)piperazin-1-yl)ethoxy)-5-oxopentanoic acid (Folic-acid modified piperazin NBD, FoPz)

To a solution of HEtPz (307 mg, 1 mmol) and folate (441 mg, 1 mmol) in dry CH_2Cl_2 was added Bis(2-oxo-3-oxazolidinyl)phosphonic chloride (50.8 mg, 0.2 mmol) and 4-dimethylamiopryidine (12.2 mg, 0.1mmol). Then the reaction mixture was stirred overnight at room temperature. The obtained precipitation was filtered and washed with CH_2Cl_2 for several times to afford the final product as yellow powder (322 mg, 0.45 mmol). Yield: 45%.

^{1}H NMR ($CDCl_3$, 600 MHz) δ (ppm) 10.91 (s, 1 H), 8.70 (s, 1H), 8.51 (d, 1 H, *J* = 9 Hz), 8.23 (s, 2 H), 8.12 (d, 2 H, *J* = 7.8 Hz), 7.62 (d, 2 H, *J* = 8.4 Hz), 6.74 (d, 1H, *J* = 9 Hz), 6.62 (d, 2 H, *J* = 8.4 Hz), 4.82 (s, 1 H), 4.52 (s, 1 H), 4.31-4.28 (m, 2 H), 4.21 (t, 2 H, *J* = 7.8 Hz), 3.75 (t, 4 H, *J* = 7.8 Hz), 3.22 (t, 4 H, *J* = 7.8 Hz), 2.28 (t, 2 H, *J* = 7.8 Hz), 2.02-1.99 (m, 2 H), 2.00-1.85 (m, 2 H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 174.6, 174.4, 167.0, 159.5, 157.6, 157.4, 153.1, 151.2, 148.4, 145.5, 145.4, 145.3, 136.9, 129.7, 128.6, 123.6, 122.1, 112.0, 105.5, 64.0, 63.9, 58.4, 55.7, 52.4, 51.3, 46.6, 46.2, 31.1, 26.6; HRMS (ESI) calcd for $C_{31}H_{32}N_{12}O_9Na$ $[M+Na]^+$: 739.2308; found: 739.2307.



Scheme S1 pH sensing process of FoPz and the photograph of 5μM FoPz in varying pH buffers (from left to right, pH=10, 8, 7, 6, 5 and 3).

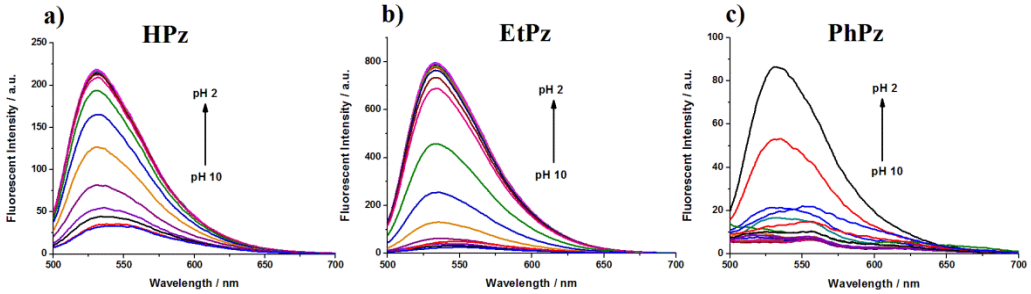


Figure S1 Fluorescent emission changes with the pH titration curve of 1 μM (a) HPz, (b) EtPz and (c) PhPz. All samples were measured in 10 mM potassium phosphate buffer at pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0. Excitation wavelength is 468 nm. Measuring temperature is 25℃.

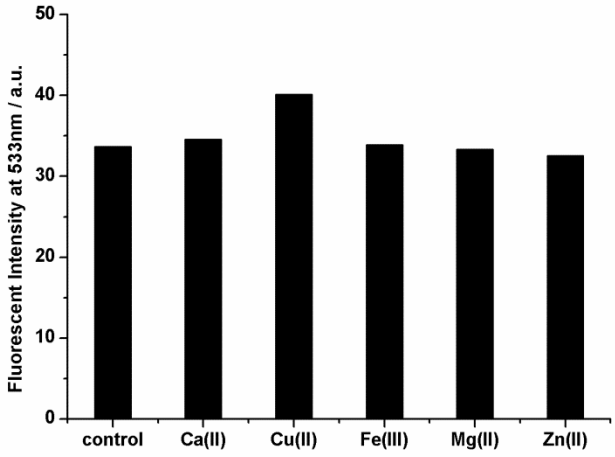


Figure S2 Fluorescence responses at 533nm of 1.0 μM FoPz to diverse metal ions in phosphate buffer (10 mM pH 7.5). The concentrations of all the cations are 50 μM and measuring temperature is 25℃.

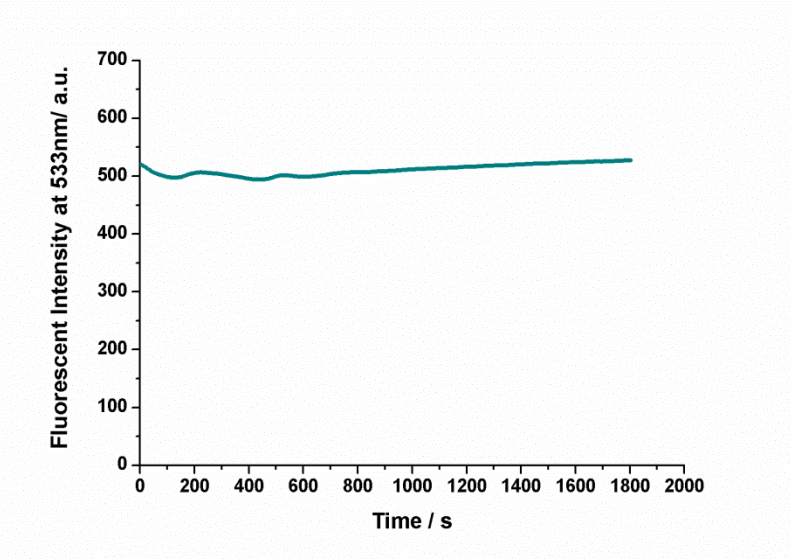


Figure S3 Time course of the FoPz were measured by spectrofluorometer (λex= 468 nm and λem = 533 nm) within 30 min. The concentration of FoPz was 1 μM in 10 mM phosphate buffer at pH 4.5.

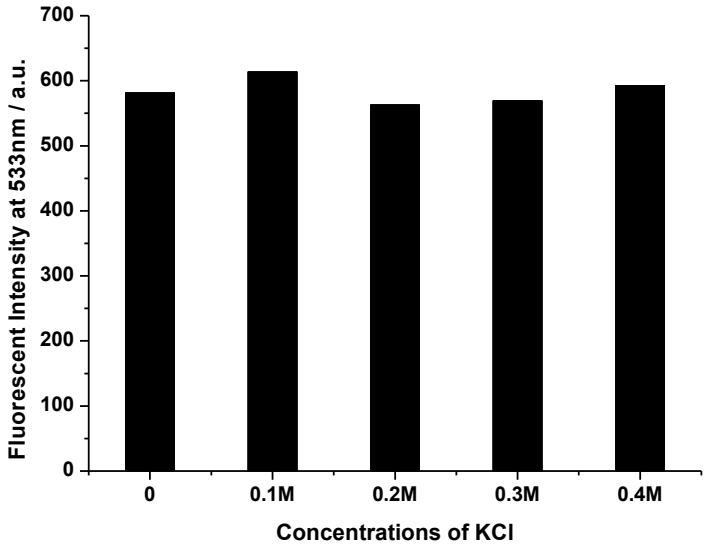


Figure S4 Effects of ion strength on the fluorescence of the FoPz were measured by spectrofluorometer (λex= 468 nm and λem = 533 nm). The concentration of FoPz was 1 μM in 10 mM phosphate buffer at pH 2. The concentration of KCl ranges from 0-0.4M.

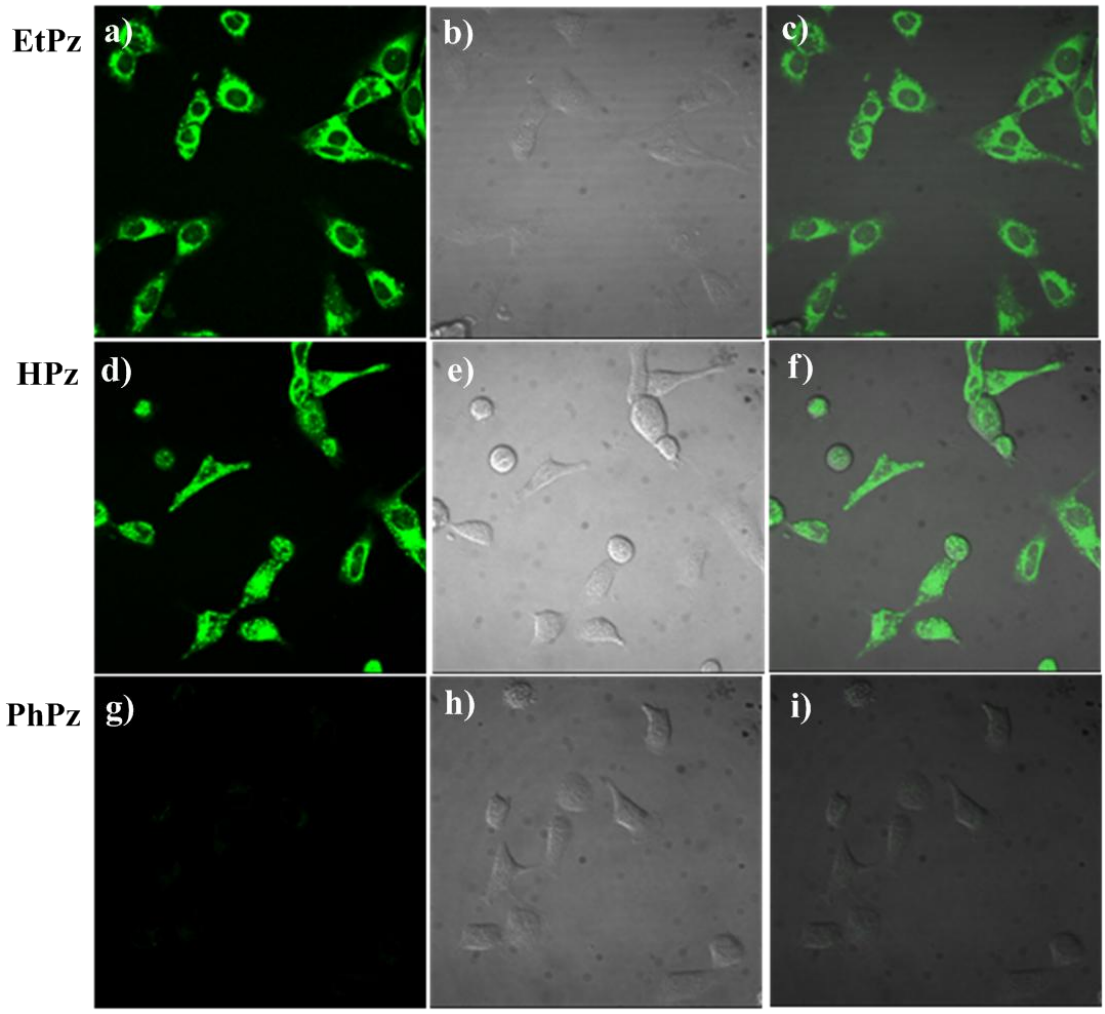


Figure S5 Confocal images of HeLa cells incubated with 5 μM EtPz (a-c), HPz (d-f) and PhPz (g-i) for 1h. Images in the left column were collected at λex=488nm. Images in the middle column were collected in the bright field. Images in the right column were merged images.

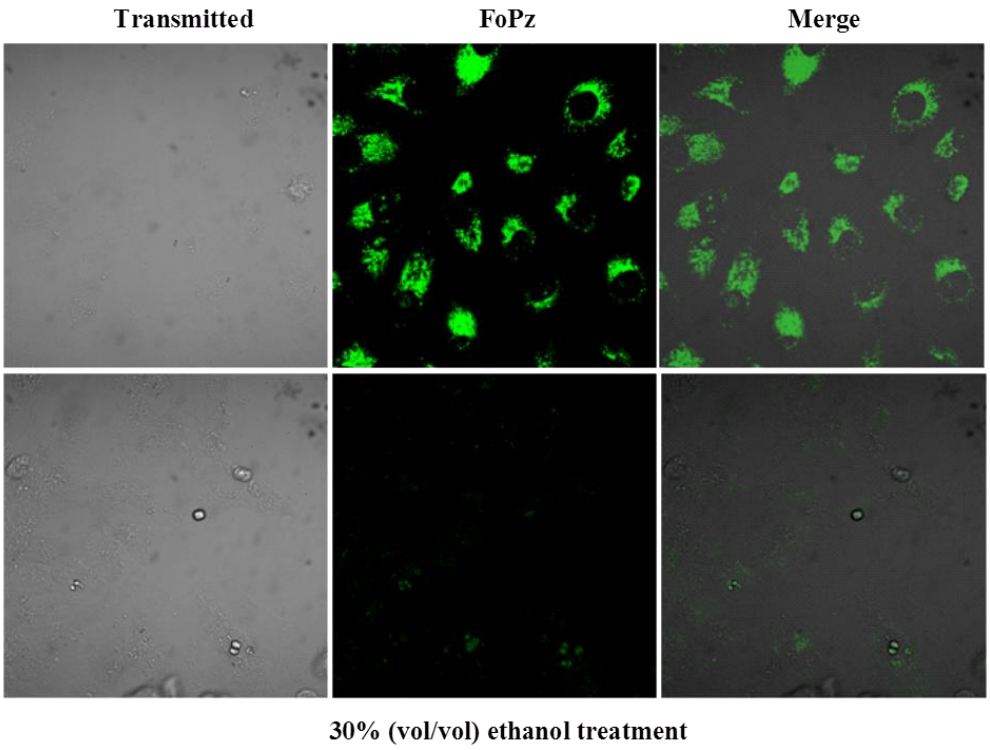


Figure S6 The activatable probe FoPz can produce a fluorescent signal only from living cells. Bright field (Left), fluorescent (middle) and merge (right) images were obtained 1h after addition of 5 μM FoPz (up row) and then after treatment with 30% ethanol for 30 min (bottom row).

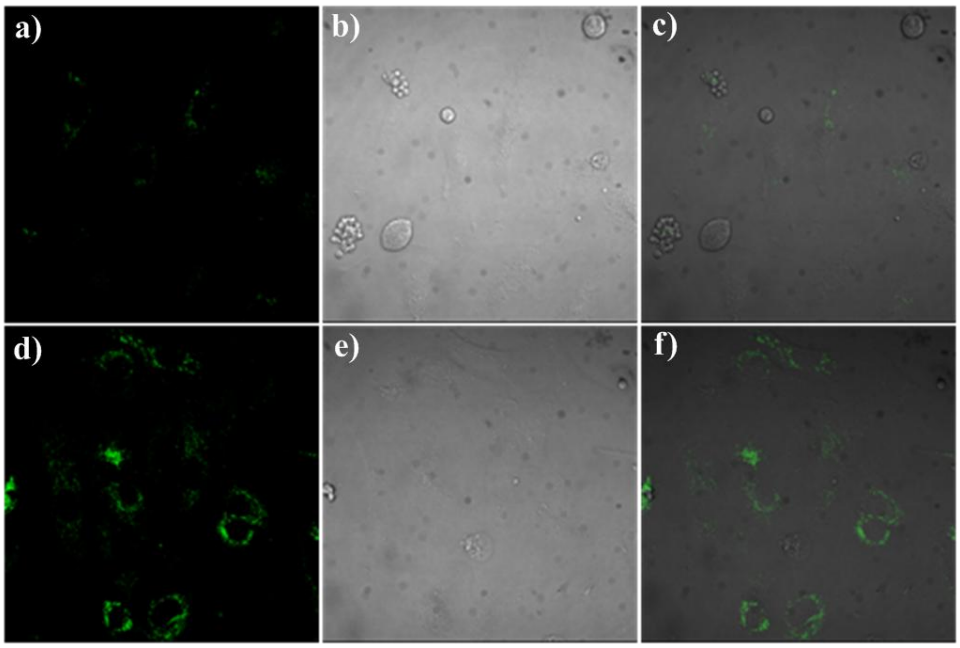


Figure S7 The activatable probe FoPz enters HeLa cells more rapidly through folate receptor mediated internalization than small molecular diffusion. a-c) HeLa cells were incubated in DMEM (with folic acid); d-f) HeLa cells were incubated in RPMI-1640 (without folic acid). Fluorescent (Left), bright field (middle) and merge (right) images were obtained 30min after addition of 5 μM FoPz.