Electronic Supplementary Information

A Novel Near-Infrared Fluorescent Probe for the Imaging of Viscosity in Cells and Tumor-Bearing Mice

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1. Experimental Section.

Reagents and instruments. Malonitrile, isophorone, 4-(dimethylamino)cinnamaldehyde, heptane, acetic acid, piperidine, Nystatin was purchased from Macklin (Shanghai, China). Nuclear magnetic resonance (NMR) spectra were measured on a Bruker Avance II NMR spectrometer (Germany). Mass spectra (MS) were acquired on a Bruker Autoflex MALDI-TOF mass spectrometer (Germany). High Resolution Mass spectra (HRMS) were acquired on a Agilent Technologies 1260II/6230 liquid chromatography mass spectrometer (USA). All fluorescence measurements were taken on a PerkinElmer LS-55 fluorescence spectrometer (USA). UV-vis absorption spectra were recorded with a PerkinElmer Lambda 25 spectrophotometer (USA). The viscosity was measured with a NDJ-5S rotational viscometer (China). Fluorescence images of cells were obtained using an Olympus FV1000 laser confocal microscope (Japan). Fluorescence imaging of mice was performed on an IVIS Lumina XR small animal optical in vivo imaging system (USA).

General procedure for fluorescence measurement. The solutions for viscosity were prepared by mixing water and glycerol in different proportions. The measurements were carried out with a viscometer, and each viscosity value was recorded. The solutions of probe IC-V with different viscosity values were prepared by adding the stock solution of IC-V (10.0 mM, 10.0 μL) in water-glycerol solution (10.0 mL) to obtain the final concentration of IC-V (10.0 μM). These solutions were sonicated for 10 min to eliminate air bubbles and allowed to stand for 1 h at a constant temperature. Then, the fluorescence spectra were recorded at excitation of 530 nm with the excitation and emission slit widths set at 5 and 5 nm, respectively. The relationship between the fluorescence emission intensity of the probe and

the viscosity of the solvent is well expressed by the Forster-Hoffmann equation,

$$\text{Log}\,(I_f) = c + x \log \eta$$

Where I_f is the fluorescence intensity, η is the viscosity of solution, and x and c are constant.

Cell incubation and fluorescence imaging. CT26 cells, HepG2 cells and 293T cells were provided by the State Key Laboratory of Chemo/Biosensing and Chemometrics of Hunan University (Changsha, China). The cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) and 10 % FBS (fetal bovine serum) in an atmosphere of 37 °C and 5% CO_2. When the cell density reached 90% of confluence, a subculture was done and the medium was changed approximately every day. The cytotoxicity of IC-V was evaluated by CCK-8 counterstaining assay done by using commercial kits purchased from Beyotime (Nantong, China).

The cells were seeded in a 20 mm glass-bottom dish and grown for 24 h before the imaging experiment. For imaging the change of viscosity, the cells were divided into three groups. The first group was control treated by IC-V (10 μM). The second group was treated by nystatin (30 μM) at 37 °C for 30 min, and then IC-V (10 μM) at 37 °C for 30 min. The third group was incubated by Cu^{2+} (30 μM) at 37 °C for 30 min, and then IC-V (10 μM) at 37 °C for 30 min. For differentiating cancer cells from normal cells, CT26 cells, HepG2 cells and 293T cells were treated with IC-V (30 μM) at 37 °C for 30 min. All the cells were washed by PBS buffer solution three times and the fluorescence image of cells were performed by confocal fluorescence microscope. The relative pixel intensity was calculated by measuring

the average intensity of each cell in the whole field using the Image J software.

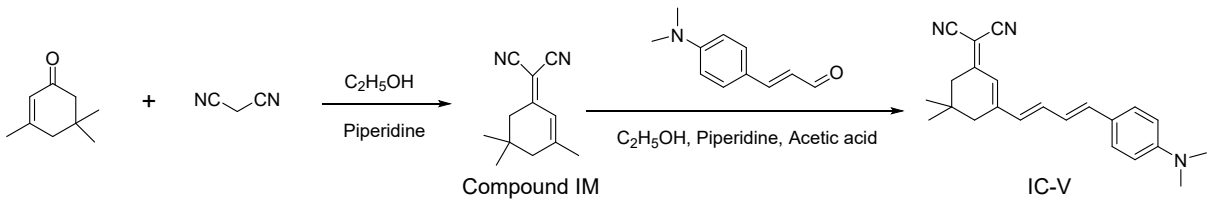
Fluorescence imaging in mice. Six-week-old male BALB/C nude mice were chosen and kindly kept in all the experimental process. All animal experiments were performed in accordance with the guidelines “Regulations of Hunan Province on the administration of experimental animals” and approved by the ethics committee at Xiangtan University. The mice were fasted for 12 h to avoid possible fluorescence interference.

For fluorescence imaging of viscosity in vivo, the normal mice were divided into four groups. The first group was blank group untreated. The second group was injected by nystatin (10 μg/mL) in abdomen for 2 days to produce viscosity increasing. The third group was offered an intraperitoneal injection of IC-V (100 μM, 100 μL). The fourth group was injected by nystatin (10 μg/mL) in the abdomen for 2 days, then offered an intraperitoneal injection of IC-V (100 μM, 100 μL).

For distinguishing normal mice and tumor-bearing mice, the normal mice were intravenously injected with IC-V (100 μM, 100 μL). The tumor-bearing mice model were established by underarm injecting with CT26 cells. They were given an intravenous injection of IC-V (100 μM, 100 μL), and the images were acquired at 0, 1, 2, 4 h. Then, the normal mice and tumor-bearing mice were sacrificed and major organs were excised, including heart, liver, spleen, lung, kidneys and tumors, washed in PBS solution and imaged.

All mice remained anesthetized throughout the imaging period. The images were acquired using the small animal in vivo optical imaging system. The excitation was set at 560 nm and the emission was set at 650-750 nm.

2. Synthesis of probes.



Scheme S1. Synthetic route for IC-V.

Compound IM. Isophorone (0.26 g, 4.0 mmol), malononitrile (0.55 g, 4.0 mmol) and piperidine (0.2 mL) were dissolved in dry ethanol (15.0 mL). The mixture was stirred for 12 h at 60 °C. After cooling to room temperature, the solution was slowly poured into water (100.0 mL) and the precipitated solid was filtered. The obtained crude product was recrystallized with heptane to afford a yellow solid. Yield: 0.60 g (80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.62 (s, 1H), 2.51 (s, 2H), 2.17 (s, 2H), 2.03 (s, 3H), 1.01 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5, 159.9, 120.6, 113.2, 112.4, 78.2, 45.7, 42.6, 32.4, 27.8, 25.4. MS (TOF): 186.2.

Probe IC-V. Compound IM (0.19 g, 1.0 mmol), 4-(dimethylamino) cinnamaldehyde (0.18 g, 1.0 mmol) and piperidine (0.1 mL) were mixed in ethanol (15 mL), and then acetic acid (0.1 mL) was added to the solution. The reaction mixture was refluxed with stirring for 12 h and then evaporated in vacuo. The mixture was purified by silica gel chromatography using petroleum ether/ CH_2Cl_2 (3:1, V/V) as the eluent to yield a brown solid (0.25 g): Yield, 73%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ 7.36 (d, J = 8.7 Hz, 2H), 6.79-6.75 (m, 2H), 6.68 (d, J = 6.5 Hz, 4H), 6.43 (d, J = 9.0 Hz, 1H), 3.02 (s, 6H), 2.54 (s 2H), 2.38 (s, 2H), 1.05 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$, ppm) δ 168.9, 154.6, 139.8, 139.3, 130.4, 128.9, 128.6, 123.8, 122.8, 121.8, 114.0, 113.2, 112.1, 76.3, 42.9, 40.2, 39.2, 31.8, 27.9. HRMS m/z: calcd for

$C_{23}H_{25}N_3$ $[M+Na]^+$: 366.1941, found: 366.1944.

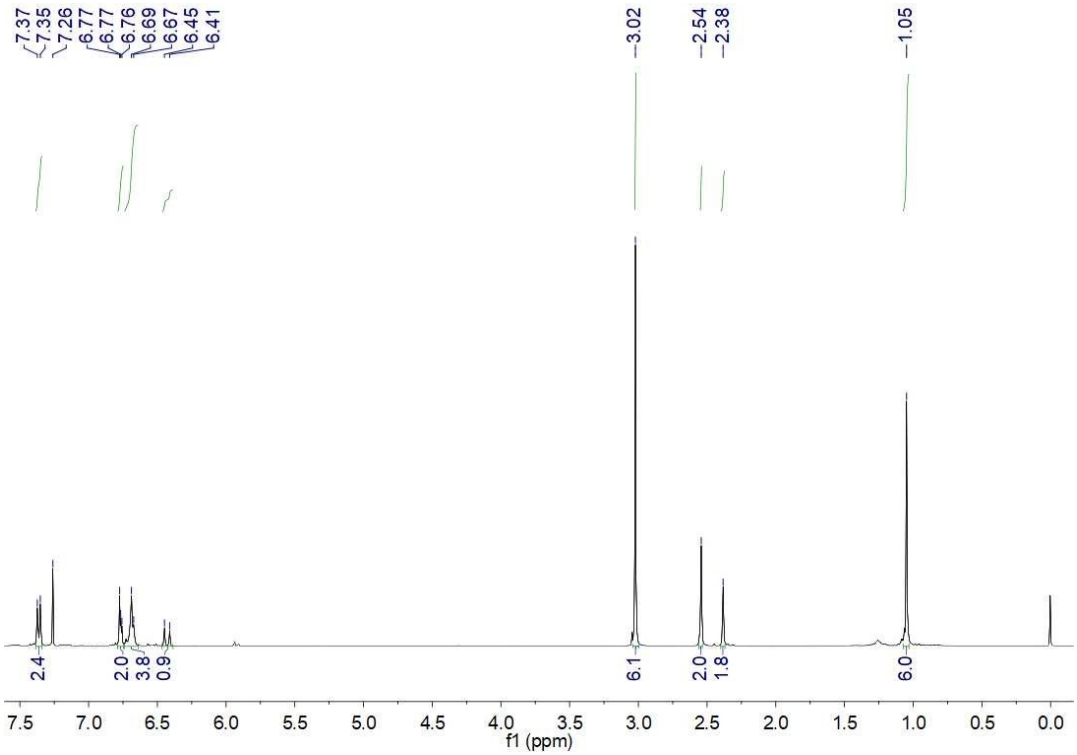


Fig. S1 ^{1}H NMR spectra of IC-V in $CDCl_3$.

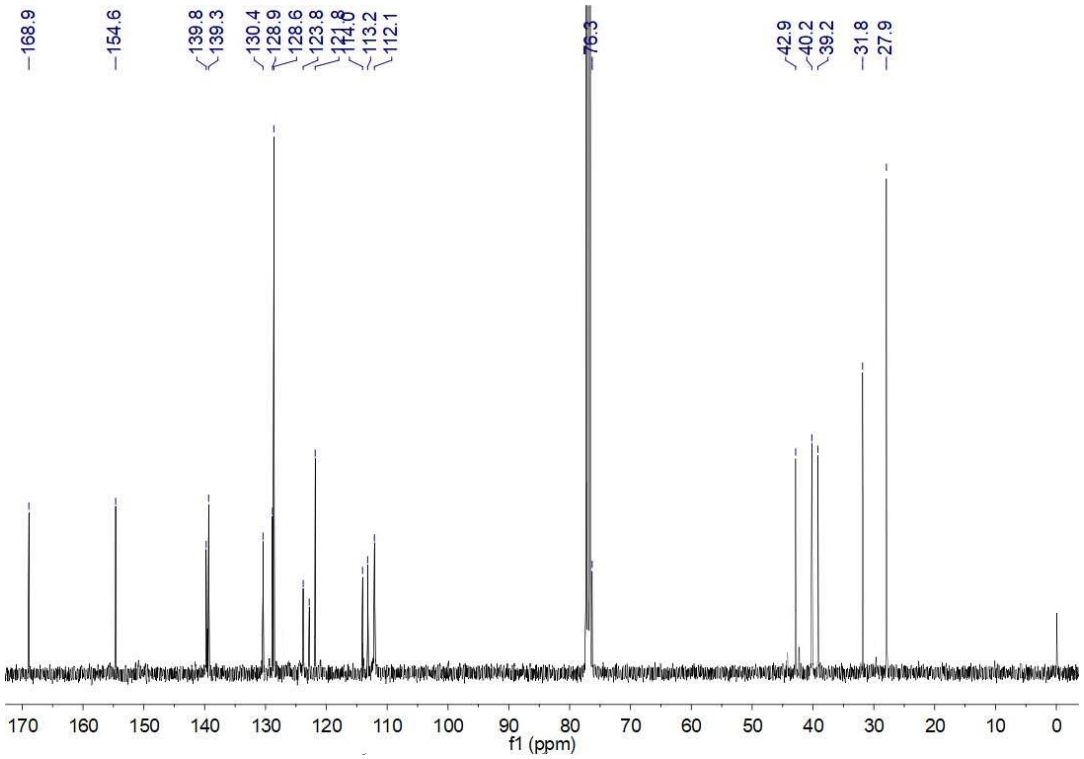


Fig. S2 ^{13}C NMR spectra of compound IC-V in $CDCl_3$.

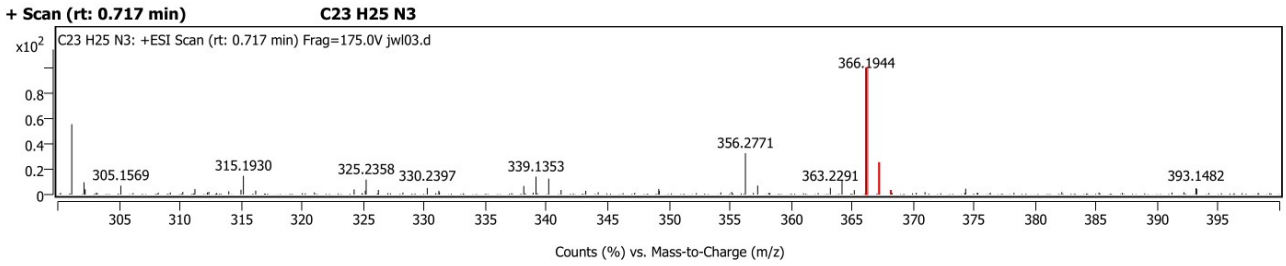
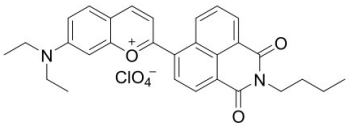
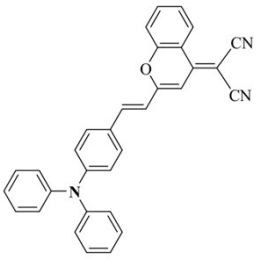
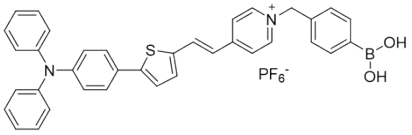
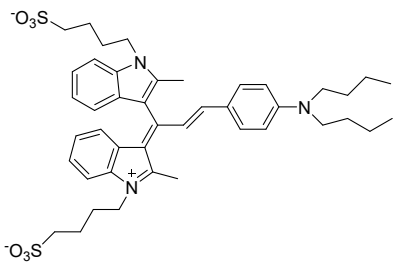
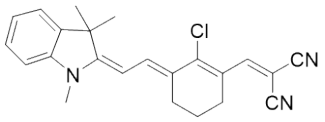
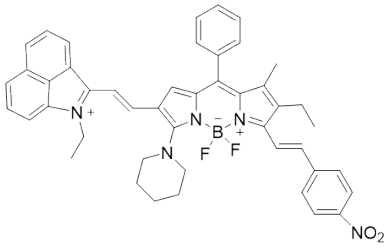
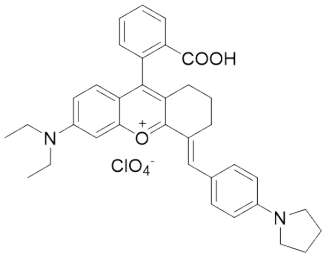
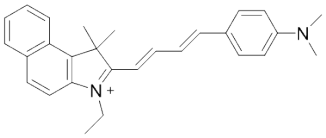
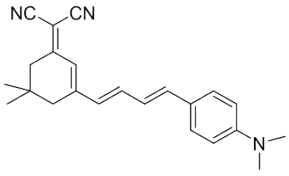


Fig. S3 HRMS spectra of compound IC-V.

3. Spectral data.

Table S1 Properties of representative fluorescent probes for viscosity

Probe	Excitation (nm)	Emission (nm)	Stoke shift (nm)	Response signal (fold)	Bioimaging applications	Ref.
	505	650	145	3	Tumor-bearing mice	19(a)
	520	696	186	30	Blood Vessel & tumor-bearing mice	19(b)
	488	666	178	--	Inflammation & cancer models.	19(c)
	670	710	40	36	Cell membranes	19(d)
	650	719	69	13.77	Cellular lipid droplets, zebra fishes & mice	19(e)
	818	982	164	31	Diabetes-induced liver injury mice	19(f)

	649	740	91	200	Mitochondrial dynamic & fatty liver mice	19(g)
	580	700	120	22	Diabetic mice	19(h)
	530	700	170	180	Tumor-bearing mice	This work

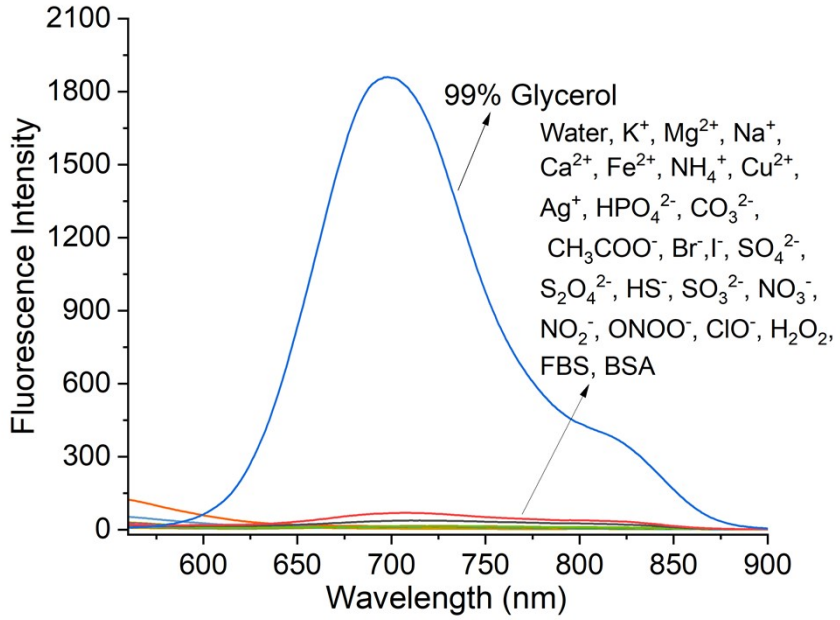


Fig. S4 Fluorescence spectra of IC-V (10 μM) in various species (200 μM). λ_{ex} = 530 nm.

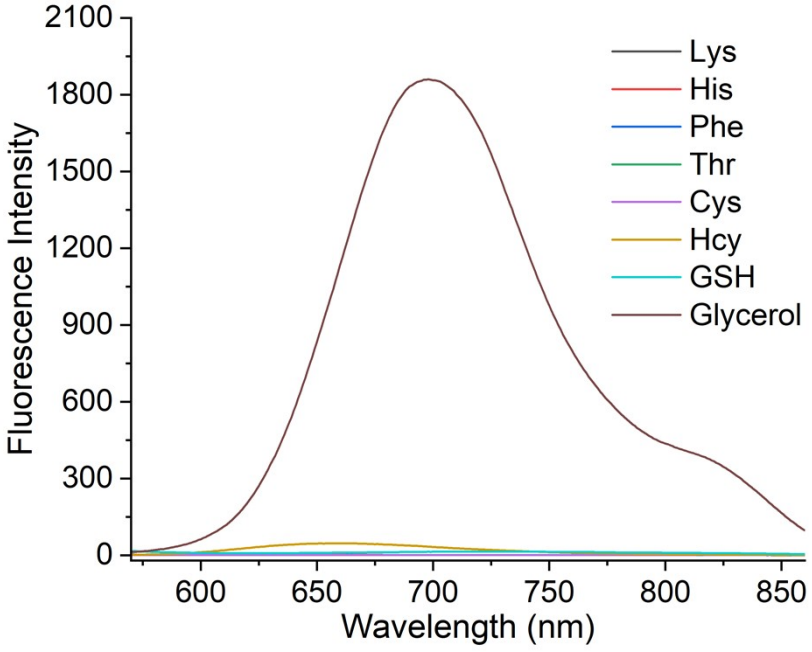


Fig. S5 Fluorescence spectra of IC-V (10 μM) in various amino acids (200 μM) (Lys, His, Phe, Thr) and biothiols (Cys, Hcy, GSH). λ_{ex} = 530 nm.

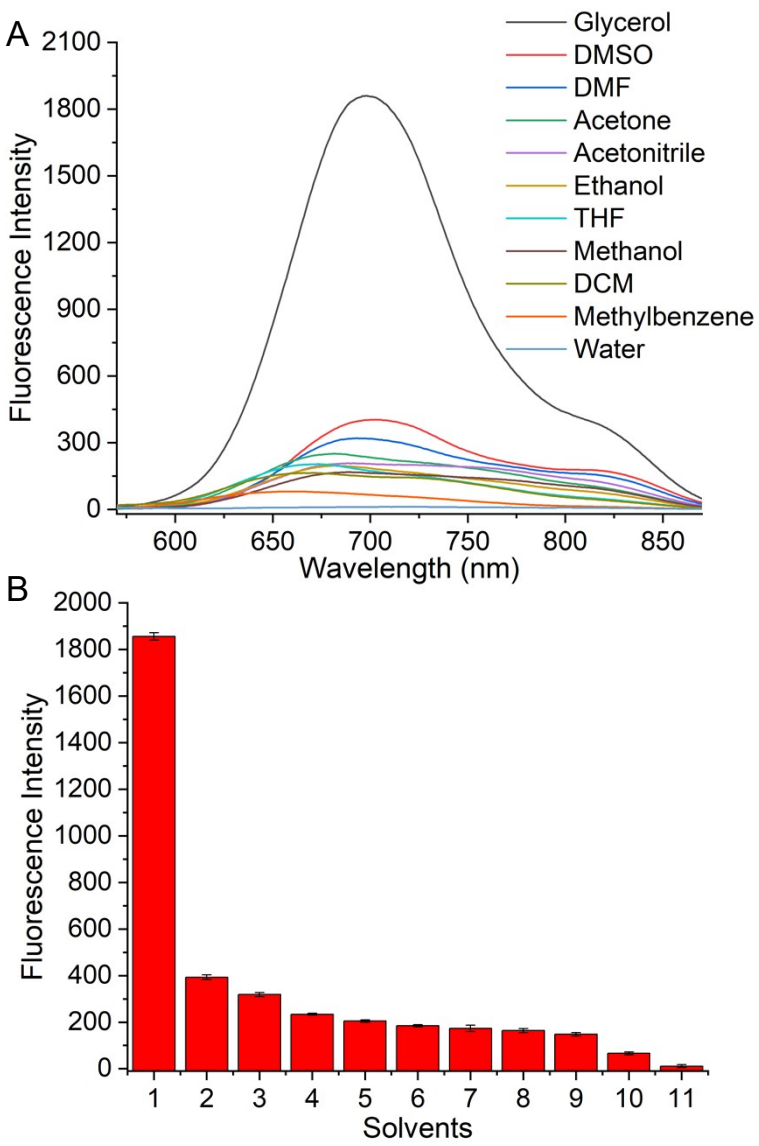


Fig. S6 (A) Fluorescence spectra and (B) fluorescence intensity of IC-V (10 μM) in various solvents of different polarity: 1. Glycerol; 2. DMSO (7.2); 3. DMF (6.4); 4. acetone (5.4); 5. acetonitrile (6.2); 6. ethanol (4.3); 7. THF (4.2); 8. methanol (6.6); 9. DCM (3.4); 10. methylbenzene (2.4); 11. water (10.2). λ_{ex} = 530 nm.

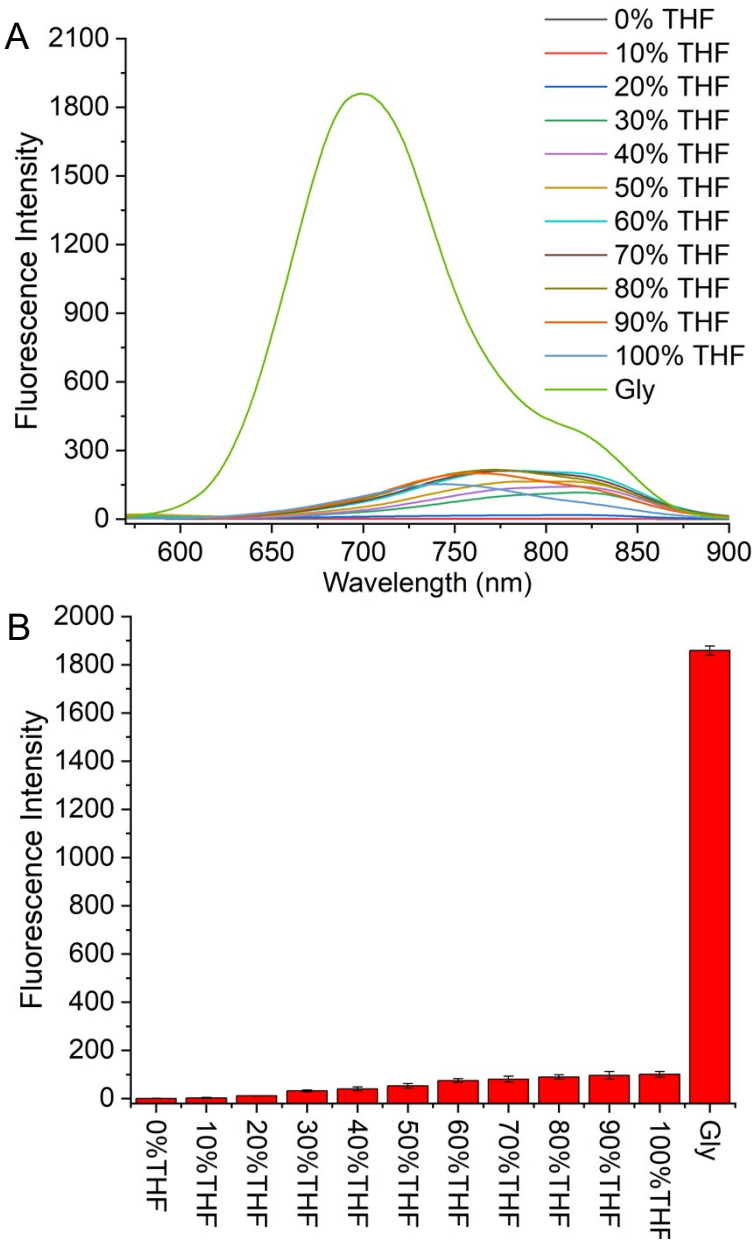


Fig. S7. (A) Fluorescence spectra and (B) fluorescence intensity of IC-V (10 μM) in glycerol and mixed solutions of THF and H_2O in different proportions. λ_{ex} = 530 nm, λ_{em} = 700 nm.

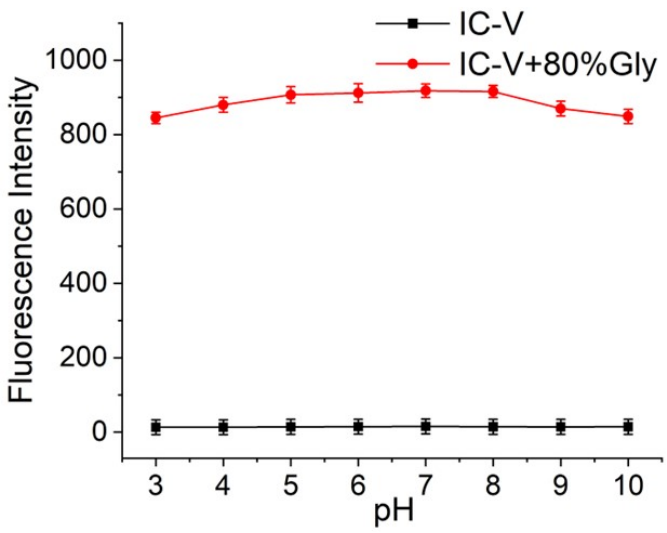


Fig. S8 Effect of pH on the fluorescence intensity of IC-V (10 μM) in glycerol and water. λ_{ex} = 530 nm. λ_{em} = 700 nm. Data are average ± SD (n = 3).

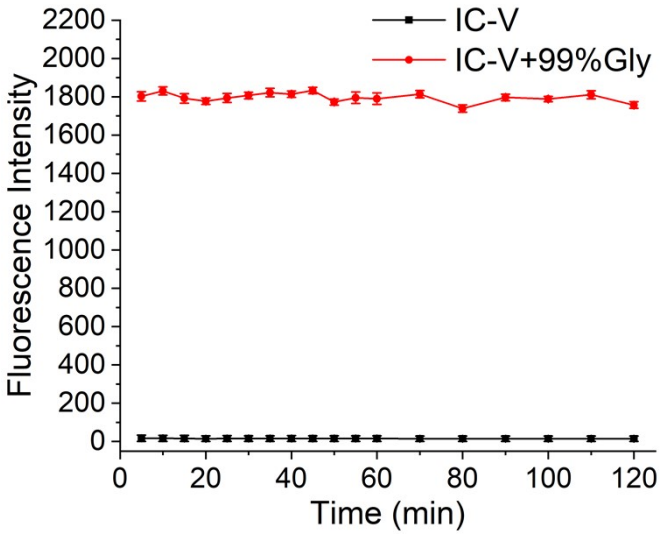


Fig. S9 Photostability of IC-V (10 μM) in glycerol and water. λ_{ex} = 530 nm. λ_{em} = 700 nm. Data are average ± SD (n = 3).

The solubility of IC-V in mixed solutions of DMSO and H_2O in different proportions was studied. As shown in the Fig. S10, the absorbance of the probe in 0.1% DMSO solution hardly decreases, while the absorbance in 0.01% DMSO solution decreases significantly, indicating that IC-V has a good solubility in 0.1% DMSO solution.

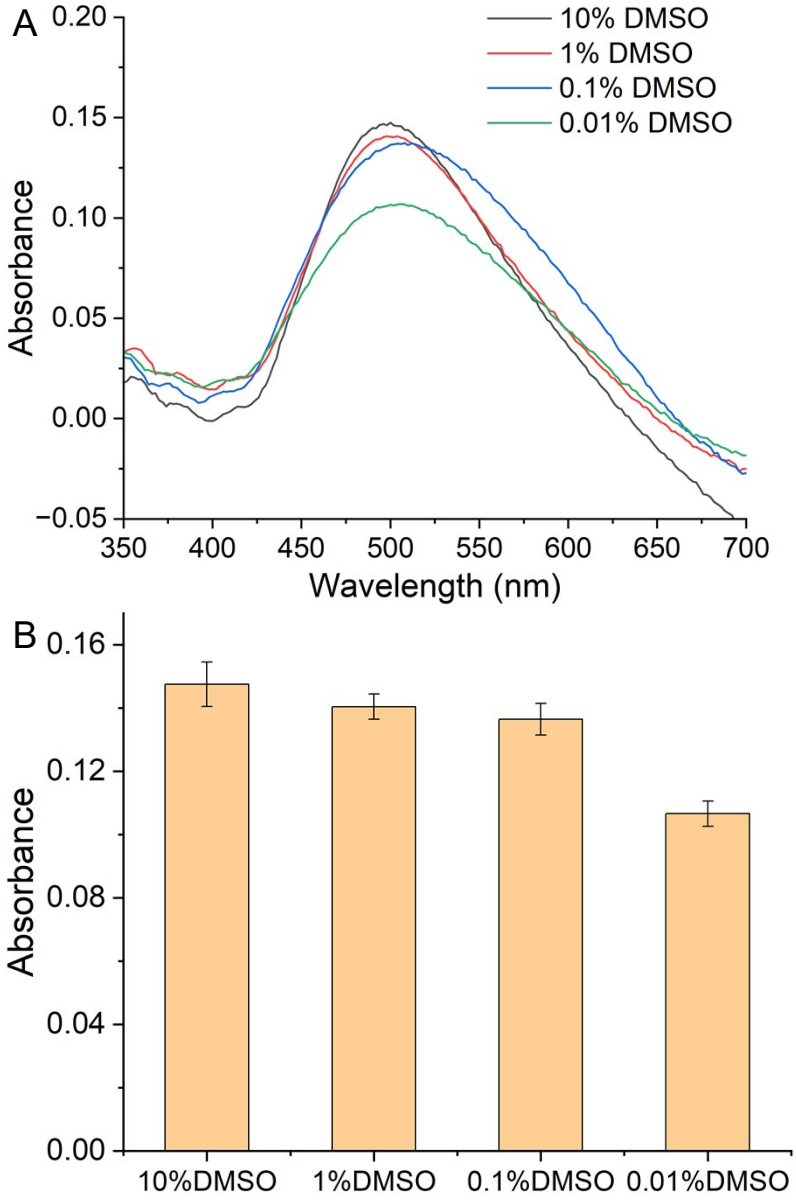


Fig. S10 (A) Absorption spectra and (B) absorbance of IC-V (10 μM) in mixed solutions of DMSO and water at different proportions.

4. Theoretical calculations.

Table S2 Detailed description for DFT calculations using Gaussian 16W software.

% Section	%mem=200MB		
Route Section	# opt b3lyp/6-311g(d,p)		
Energies	2.51 eV		
Molecule Specification			
C	-2.88700000	-4.21350000	0.77990000
C	-4.07680000	-3.43350000	0.20070000
C	-3.84020000	-1.94090000	0.48420000
C	-2.46340000	-1.47450000	0.05380000
C	-1.45510000	-2.36810000	-0.02840000
C	-1.55590000	-3.67570000	0.29010000
C	-0.49120000	-4.50110000	0.19280000
C	-5.38590000	-3.89060000	0.87810000
C	-2.21210000	-0.17870000	-0.24050000
C	-4.19770000	-3.68440000	-1.31810000
C	-3.09590000	0.83370000	-0.15790000
C	-2.79560000	2.11440000	-0.43790000
C	-3.58680000	3.20440000	-0.38730000
C	-4.92450000	3.36520000	-0.28690000
C	-5.41830000	4.59480000	-0.04340000
C	-6.73470000	4.83470000	0.07570000
C	-7.67340000	3.87540000	-0.06620000
C	-7.17150000	2.65810000	-0.35900000
C	-5.85560000	2.41320000	-0.47180000
N	-8.93050000	4.10060000	0.04890000
C	-9.89870000	2.98660000	-0.13180000
C	-9.40490000	5.47170000	0.37110000
C	0.69670000	-4.10460000	-0.22180000
N	1.74380000	-3.76560000	-0.58550000
C	-0.58620000	-5.77400000	0.52480000
N	-0.67300000	-6.89190000	0.81890000
H	-2.88520000	-4.13760000	1.89290000
H	-3.02660000	-5.28750000	0.52060000
H	-3.93760000	-1.73300000	1.57580000
H	-4.65240000	-1.39370000	-0.04550000
H	-0.47370000	-1.99000000	-0.36120000
H	-5.35240000	-3.73600000	1.98030000
H	-6.26160000	-3.32360000	0.48860000
H	-5.57780000	-4.97270000	0.69860000
H	-1.19170000	0.09100000	-0.56790000
H	-4.35780000	-4.76430000	-1.53650000

H	-5.05850000	-3.12770000	-1.75270000
H	-3.28770000	-3.36680000	-1.87340000
H	-4.10290000	0.60110000	0.19870000
H	-1.73320000	2.31760000	-0.67570000
H	-3.00290000	4.14510000	-0.45060000
H	-4.73710000	5.45420000	0.09370000
H	-6.99870000	5.88090000	0.29950000
H	-7.82270000	1.78750000	-0.54020000
H	-5.58980000	1.39230000	-0.77720000
H	-9.73950000	2.18040000	0.62000000
H	-10.94230000	3.33910000	0.02330000
H	-9.86630000	2.58450000	-1.17010000
H	-10.51410000	5.49830000	0.45050000
H	-9.02350000	5.81160000	1.36080000
H	-9.14100000	6.19360000	-0.43490000

5. Biological assays.

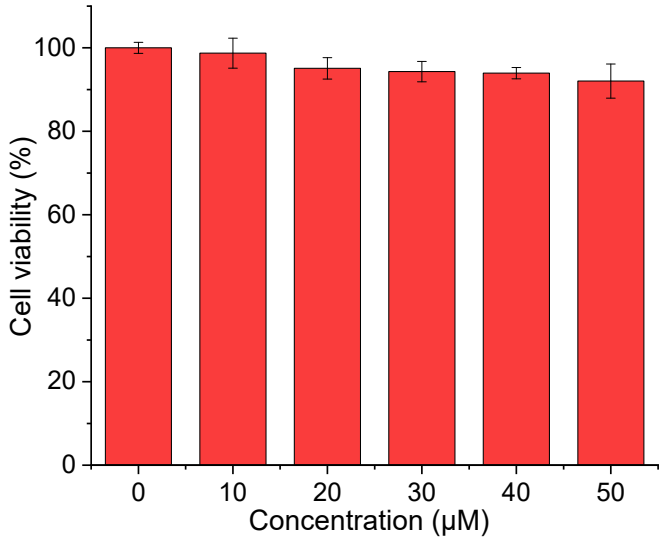


Fig. S11 Effects of the probe IC-V with varied concentrations (0-50 μM) on the viability of the CT26 cells. The probe with varied concentrations was incubated with the cells for 24 h. The viability of the cells in the absence of the probe is defined as 100 %, and the data are the mean standard deviation of five separate measurements.

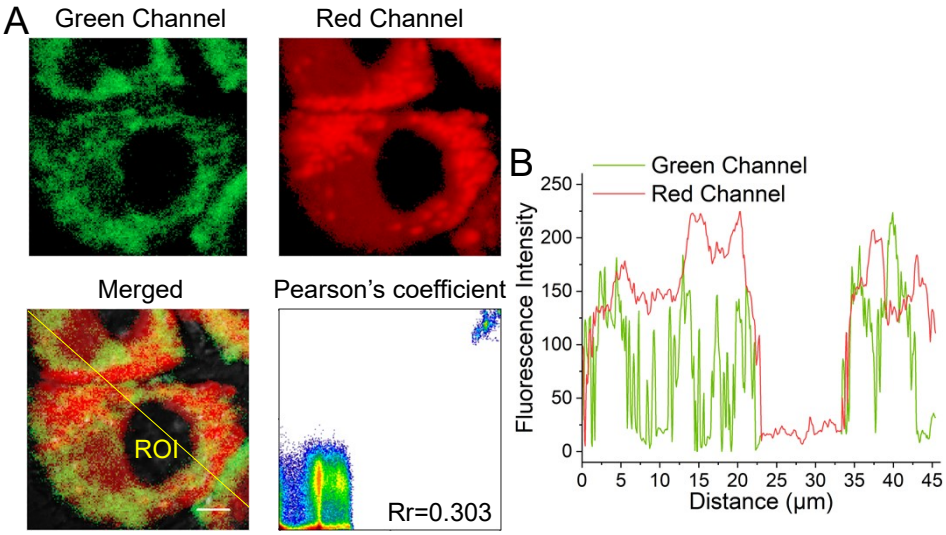


Fig. S12 (A) Colocalization images of mitochondria in HepG2 cells. (A) The cells were stained with probe IC-V and Mito-Tracker Green. Green Channel: fluorescence images of Mito-Tracker Green (λ_{ex} = 488 nm; λ_{em} =500–550 nm). Red Channel: fluorescence images of IC-V (λ_{ex} = 560 nm; λ_{em} =700–775 nm). Merge: the merged images of green channel and red channel. Scatter Plot: the scatter plot of green channel and red channel. (B) Fluorescence intensity of the regions of interest (ROI). Scale bar: 5 μm.

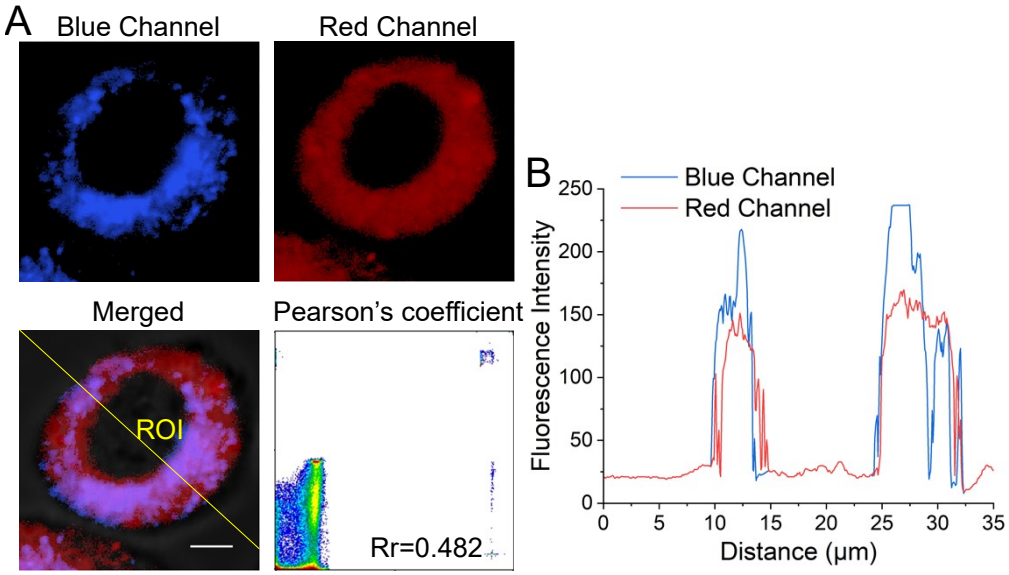


Fig. S13 (A) Colocalization images of lysosome in HepG2 cells. (A) The cells were stained with probe IC-V and Lyso-Tracker Green. Blue Channel: fluorescence images of Lyso-Tracker Green (λ_{ex} = 405 nm; λ_{em} =500–550 nm). Red Channel: fluorescence images of IC-V (λ_{ex} = 560 nm; λ_{em} =700–775 nm). Merge: the merged images of blue channel and red channel. Scatter Plot: the scatter plot of blue channel and red channel. (B) Fluorescence intensity of the regions of interest (ROI). Scale bar: 5 μm.

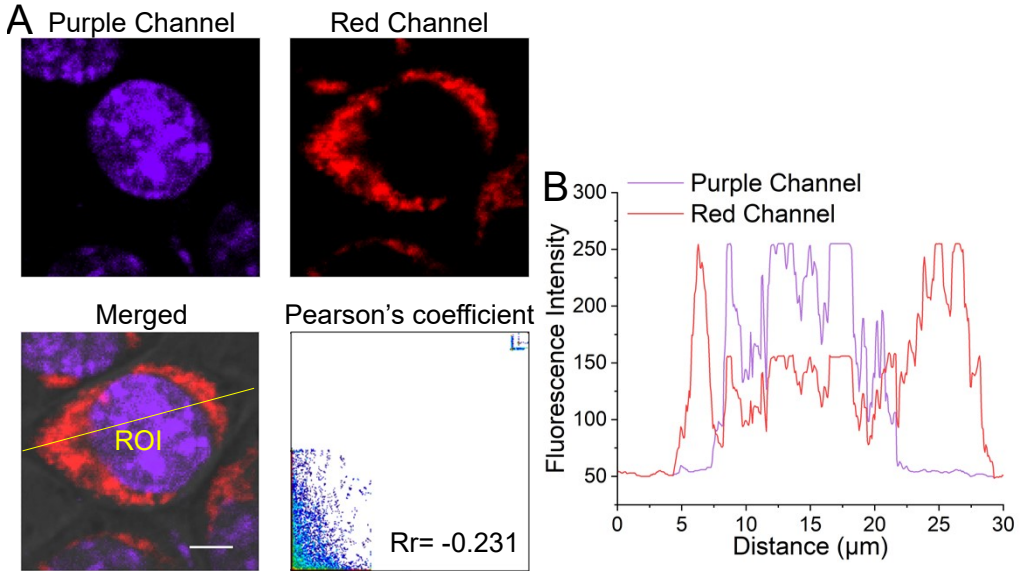


Fig. S14 (A) Colocalization images of nucleus in HepG2 cells. (A) The cells were stained with probe IC-V and Nucle-Tracker DAPI. Purple Channel: fluorescence images of Nucle-Tracker DAPI (λ_{ex} = 405 nm; λ_{em} = 425-475 nm). Red Channel: fluorescence images of IC-V (λ_{ex} = 560 nm; λ_{em} =700–775 nm). Merge: the merged images of purple channel and red channel. Scatter Plot: the scatter plot of purple channel and red channel. (B) Fluorescence intensity of the regions of interest (ROI). Scale bar: 5 μm.

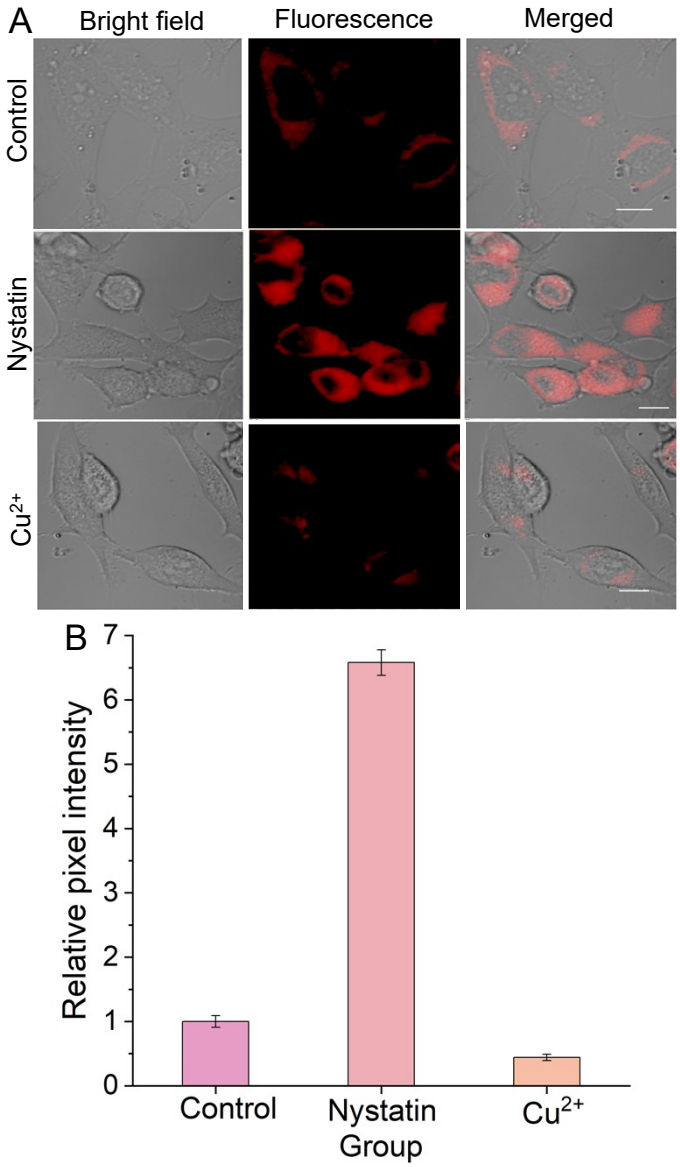


Fig. S15 (A) Fluorescence images of the viscosity in CT26 cells. Control group: the cells were incubated with probe IC-V (10 μM). Nystatin group: the cells were stimulated with nystatin (30 μM) and then incubated with probe IC-V (10 μM) for 30 min. Cu^{2+} group: the cells were treated by Cu^{2+} (30 μM) and then probe IC-V (10 μM) for 30 min. (B) Relative pixel intensity. λex = 560 nm, λem = 650-750 nm; Scale bar:10 μm. Data are average ± SD (n = 3).

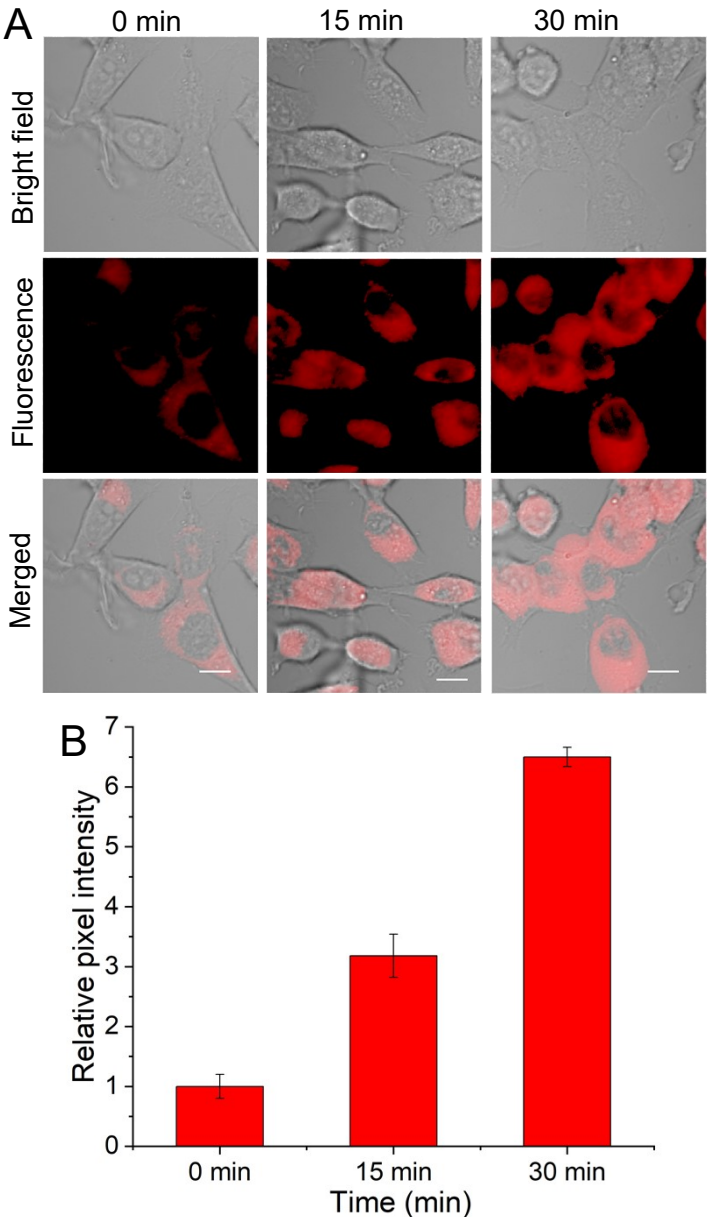


Fig. S16 (A) Fluorescence images of CT26 cells induced by nystatin (30 μM) for 30 min and then treated with IC-V (10 μM) for (0, 15, 30 min). (B) Relative pixel intensity. λ_{ex} = 560 nm, λ_{em} = 650-750 nm; Scale bar:10 μm. Data are average ± SD (n = 3).

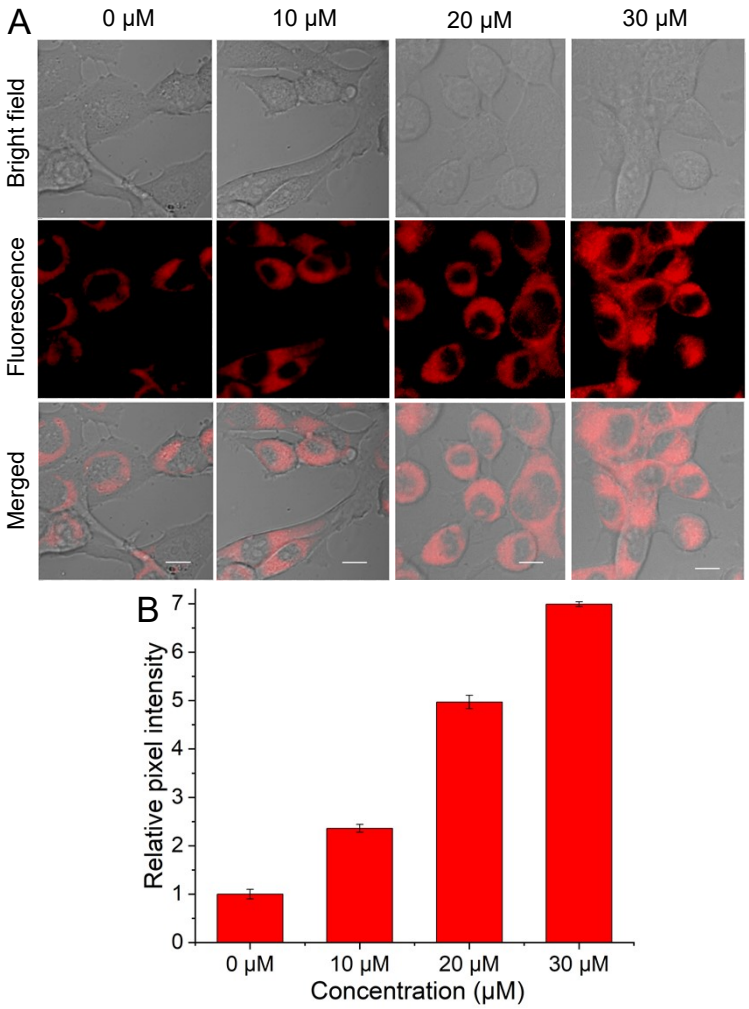


Fig. S17 (A) Fluorescence images of CT26 cells induced by nystatin (0, 10, 20, 30 μM) for 30 min and then treated with IC-V (10 μM) for 30 min. (B) Relative pixel intensity. λ_{ex} = 560 nm, λ_{em} = 650-750 nm, Scale bar:10 μm. Data are average ± SD (n = 3).

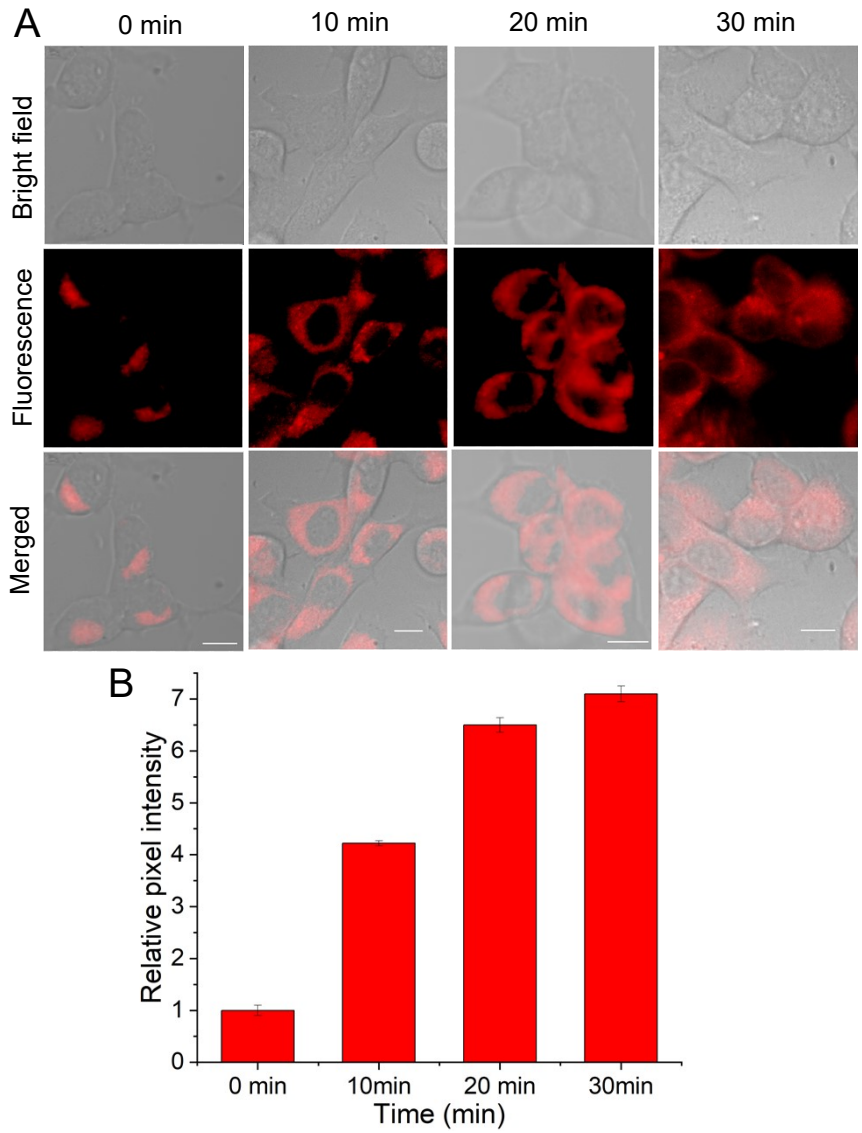


Fig. S18 (A) Fluorescence images of CT26 cells induced by nystatin (30 μM) for 0, 10, 20, 30 min and then treated with IC-V (10 μM) for 30 min. (B) Relative pixel intensity. λ_{ex} = 560 nm, λ_{em} = 650-750 nm; Scale bar:10 μm. Data are average ± SD (n = 3).

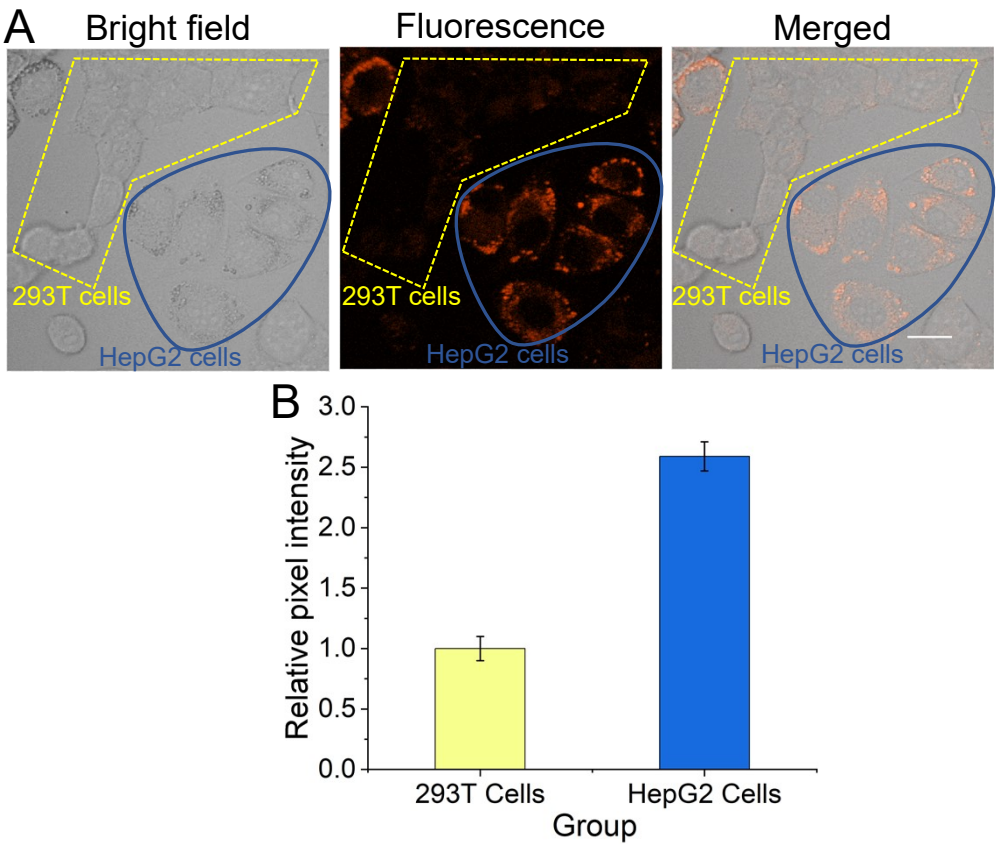


Fig. S19 (A) Fluorescence imaging of 293T cells (yellow circle) and HepG2 cells (blue circle) co-cultivated in the same dish and stained by IC-V. (B) Relative pixel intensity of the two cells. λ_{ex} = 560 nm, λ_{em} = 650-750 nm; Scale bar:20 μm. Data are average ± SD (n = 3).

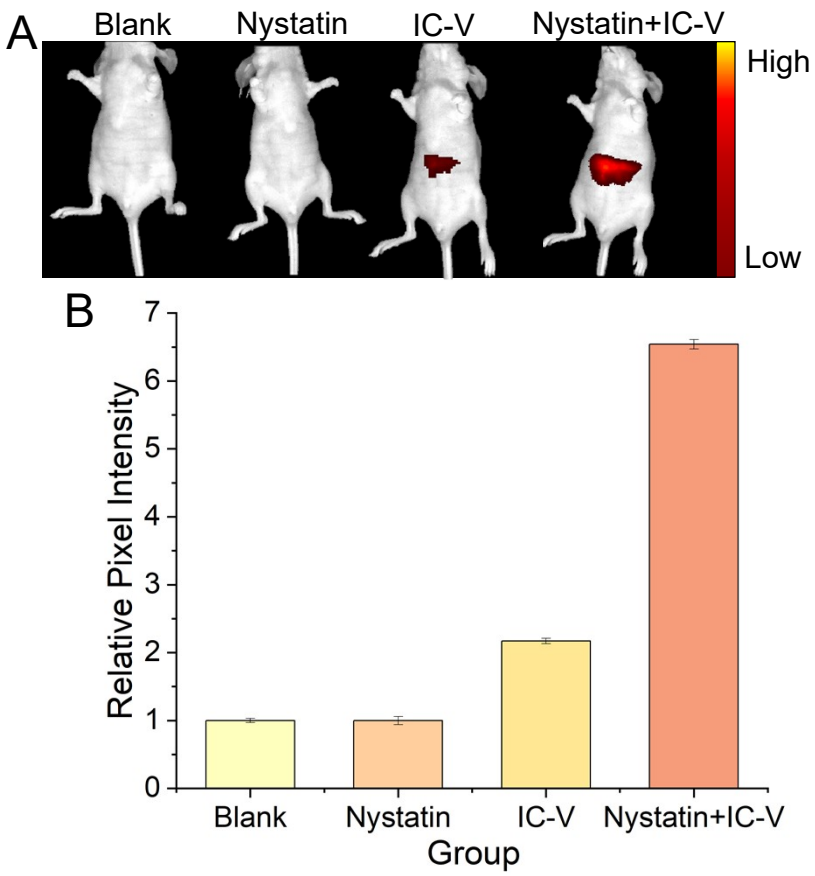


Fig. S20 (A) Fluorescence imaging of viscosity changes in normal mice. Blank group: the mice were untreated. Nystatin group: the mice were injected by nystatin (10 μg/mL) in abdomen for 2 days. IC-V group: the mice were provided an intraperitoneal injection of IC-V (100 μM) for 30 min. Nystatin+ IC-V group: the mice were injected by nystatin in abdomen for 2 days, then provided an intraperitoneal injection of IC-V (100 μM) for 30 min. (B) Relative pixel intensity in (A). λ_{ex} = 560 nm, λ_{em} = 650-750 nm. Data are average ± SD (n = 3).

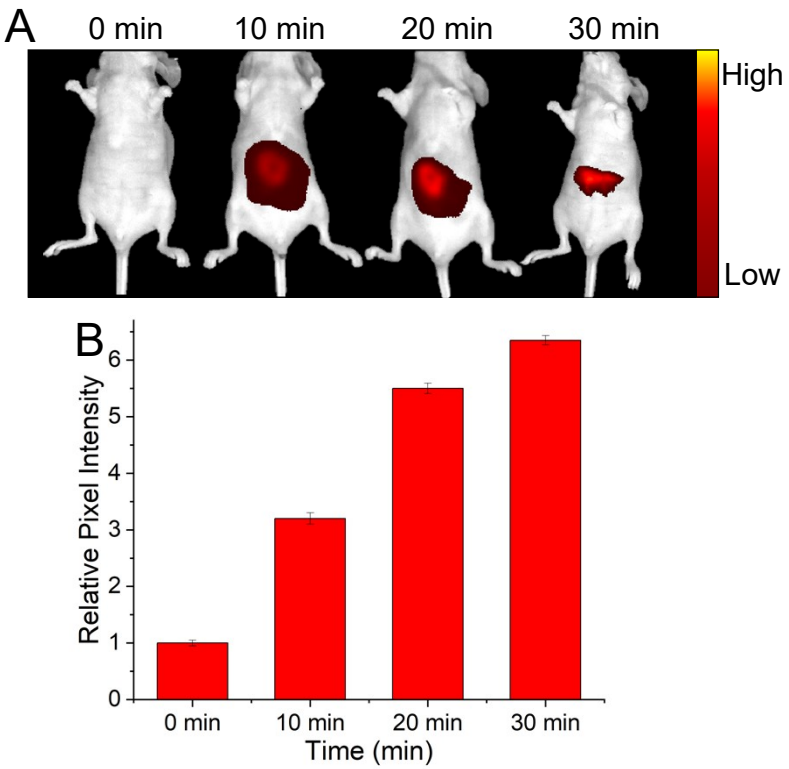


Fig. S21 (A) Fluorescence imaging of normal mice injected by nystatin (10 μg/mL) in abdomen for 2 days, and then injected intraperitoneally by IC-V (100 μM) with time (0, 10, 20, 30 min). (B) Relative pixel intensity in (A). λex = 560 nm, λem = 650-750 nm. Data are average ± SD (n = 3).

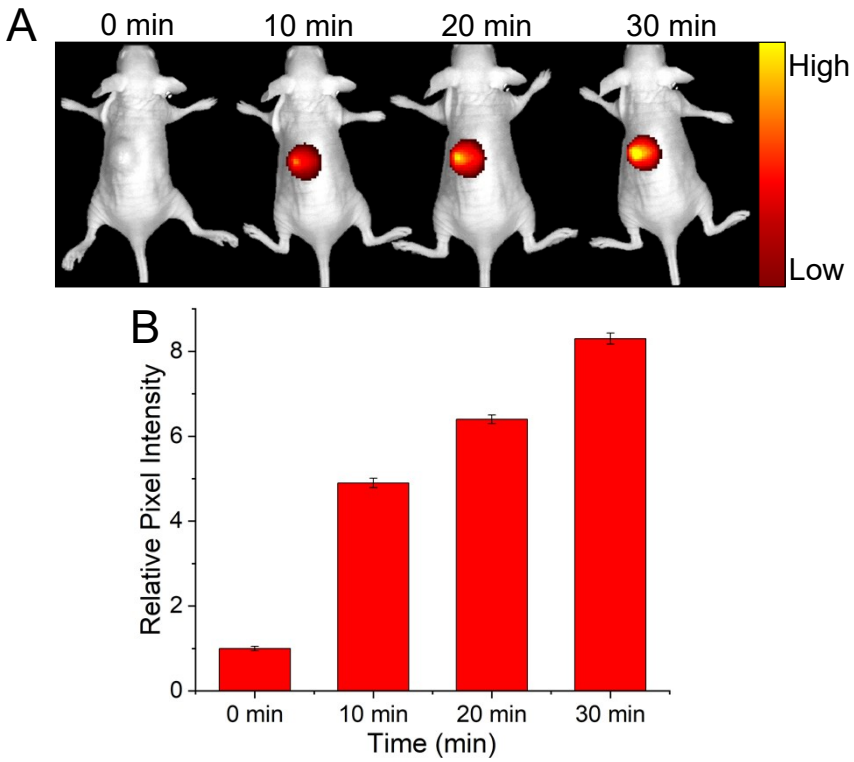


Fig. S22 (A) Fluorescence imaging of tumor-bearing mice injected intratumorally by IC-V (100 μM) with time (0, 10, 20, 30 min). (B) Relative pixel intensity in (A). λ_{ex} = 560 nm, λ_{em} = 650-750 nm. Data are average ± SD (n = 3).