Supporting Information to Accompany "**Designing Two-Photon Fluorescent Probes Based on Target-Induced Enhancement of Absorption Cross Section**"

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Synthesis of 2-(4'-*N,N*-diethylamino-2'-hydroxyphenyl)benzimidazole (1c).

The synthesis of **1c** was modified by previous literature[1]. Briefly, 4-Diethylamino-2-hydroxybenzaldehyde (0.48 g, 2.5 mmol) in 2 mL DMF and o-phenylenediamine (0.27 g, 2.5 mmol) was added. Above mixture was stired at room temperature for a few minutes, followed by the addition of sodium metabisulfite (0.48 g, 2.5 mmol) dissolved in 0.5mL H_2O in portions. The reaction was stirred at 90 °C for 4 h and poured into 30mL ice water. The precipitate was filtered and then recrystallized by ethanol as pale yellow powder (0.6 g, 85%). **^{1}H NMR** (d_6-DMSO, 300MHz), δ: 1.13 (t, 6H, *J*=6.0 Hz), 3.38 (m, 4H, *J*=6.0 Hz), 6.19 (s, 1H), 6.36 (d, 1H, *J*=3.0 Hz), 7.19 (t, 2H, *J*=3.0 Hz), 7.54 (s, 2H), 7.78 (d, 1H, *J*=9.0 Hz), 12.8 (s, 2H). HRMS (MALDI): calcd for $C_{17}H_{20}ON_3$ $[M + H]^+$ 282.1600 found 282.1601.

Synthesis of 2-(4'-methoxyl-2'-hydroxyphenyl)benzimidazole (1b).

1b was synthesized with similar procedure of **1c** by the condensation of 4-methoxyl-2-hydroxybenzaldehyde and o-phenylenediamine. **^{1}H NMR** (d_6-DMSO, 400MHz), δ: 3.38 (d, 3H, *J*=6.0 Hz), 6.61 (t, 2H, *J*=4.0 Hz), 7.25 (d, 2H, *J*=6.0 Hz), 7.62 (s, 2H), 7.54 (s, 2H), 7.96 (d, 1H, *J*=4.0 Hz). HRMS (MALDI): calcd for $C_{14}H_{13}O_2N_2$ $[M + H]^+$ 241.0971 found 241.0971.

Synthesis of 2a.

2-(4'-*N,N*-diethylamino-2'-hydroxyphenyl)benzimidazole (0.14 g, 0.05 mmol) was dissolved in 20 mL of CH_2Cl_2 and then several drops of triethylamine were added as catalyst. The mixture was stirred at room temperature for 15 min. 2, 4-Dinitrobenzenesulfonyl chloride (0.13 g, 0.05 mmol) was added and the reaction mixture was stirred at room temperature for 24h. Then the solvent was removed under reduced pressure and dark red solid was given and then collected and dried. The crude product was purified by column chromatography on silica gel (mineral ether: ethyl acetate=2:1) to give **2a** as dark red powder (0.1 g, 39%). **^{1}H NMR** (d_6-DMSO, 300MHz), δ: 1.07 (t, 6H, *J*=4.5 Hz), 3.44 (m, 4H, *J* = 6.0 Hz), 6.09 (d, 2H, *J* = 9.0 Hz), 6.78 (d, 1H, *J* = 6.0 Hz), 7.14 (d, 1H, *J* = 6.0 Hz), 7.28-7.37 (m, 4H, *J* = 9.0 Hz), 7.28 (d, 1H, *J* = 9.0 Hz), 8.23 (d, 1H, *J* = 9.0 Hz), 8.53 (d, 1H, *J* = 6.0 Hz), 9.03 (s, 1H). HRMS (MALDI): calcd for $C_{23}H_{22}O_7N_5S$ $[M + H]^+$ 512.1235 found 512.1235.

Synthesis of 2b.

The reference molecule **2b** was synthesized with the same procedure of **2a** through the reaction of **1c** and p-toluenesulfonyl chloride. **^{1}H NMR** (d_6-DMSO, 400MHz), δ: 1.08 (t, 6H, *J*=8.0 Hz), 2.19 (s, 3H), 3.33 (m, 4H, *J*=7.2 Hz), 6.36 (d, 1H, *J*=1.2 Hz), 6.75 (m, 1H, *J*=9.2 Hz), 7.03 (d, 2H, *J*=8.0 Hz), 7.15 (m, 2H, *J*=5.6 Hz), 7.45 (d, 4H, *J*=8.4 Hz), 7.66 (d, 1H, *J*=8.8 Hz). HRMS (MALDI): calcd for $C_{24}H_{26}O_3N_3S$ $[M + H]^+$ 436.1675 found 436.1689.

Spectroscopic measurements.

Absorption spectra were recorded on Shimadzu UV-2550 UV-Vis spectrophotometer (Tokyo, Japan), and one-photon fluorescence spectra were obtained with Shimadzu RF-5301 PC fluorometer (Tokyo, Japan) using a 1 cm standard quartz cell. The fluorescence quantum yield was determined by using quinine sulfate in 0.1M sulfuric acid as the reference with the literature method.[2] Two-photon fluorescence spectra were excited by mode-locked Ti:Sapphire femtosecond pulsed laser (Chameleon Ultra I, Coherent Inc.) and recorded by DCS200PC photon counting with Omno-λ5008 monochromator (Beijing Zolix Instruments Co., Ltd.).

Water solubility.

Small amount of **2a** was dissolved in DMSO to prepare the stock solutions (1.0×10^{-2} M). The solution was diluted to ($1.0 \times 10^{-7} \sim 1.0 \times 10^{-4}$) M in 3.0 mL HEPES buffer (10 mM, pH 7.4). In all cases, the concentration of DMSO in HEPES buffer was maintained to be 0.2 %.[3] The plot of fluorescence intensity against the **2a** concentration were linear at low concentration and showed downward curvature at higher concentrations (Fig. S2a). The maximum concentration in the linear region was taken as the solubility. The solubility of **2a** in buffer was ~ 10 μM (Fig. S2b).

Computational results

Geomertries of **1c**, **2a**, and **2b** were fully optimized by using B3LYP/def2-SVP level in Orca 3.0.2 program. Caculated frontier orbital energies were summarized in Table S3. The distribution of frontier orbitals and energy diagram of **1c**, **2a**, and **2b** were drawn in Fig. S6-7.

Measurement of two-photon absorption cross section.

The two-photon absorption cross section (δ) was determined by using femto second (fs) fluorescence measurement technique as described.[4] Three samples, **1c**, **2a** and **2b** were dissolved in DMSO and the two-photon induced fluorescence intensity was measured at 720−760 nm by using rhodamine B as the reference, whose two-photon property has been well characterized in the literature.[5] The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The two-photon cross section was calculated by using $\delta = \delta_r(S_s\Phi_r\phi_r c_r)/(S_r\Phi_s\phi_s c_s)$, where the subscripts *s* and *r* stand for the sample and reference molecules, respectively. The intensity of the signal collected by a CCD detector was denoted as *S*. Φ is the fluorescence quantum yield. ϕ is the overall fluorescence collection efficiency of the experimental apparatus which can be approximated by refractive index of the solvent. The number density of the molecules in solution was denoted as *c*. δ_r is the 2PA cross section of the reference molecule.

Cytotoxicity assay. MTT test was performed referred to the protocol[6] with minor change. HeLa cells were seeded in 96-well plates and incubated with different concentrations of **2a** (5, 10, and 15 μM, containing 1% DMSO), and control group added the same volume of Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific) and that containing 1% DMSO (denoted as Mock and DMSO, respectively). The experiment and control groups were incubated in an atmosphere of 5/95 (v/v) of CO_2/air at 37 °C for 24h. Next, 20 μL 5.0 mg/mL MTT solution was added into each well, followed by incubation for 4h under the same condition. Then the 100 μL supernatant were removed and added 150 μL DMSO. After shaking for 10min, the absorbance at 490 nm was measured by microplate reader (Synergy 2, BioTek Instruments Inc.). Cell survival rate was calculated by $A/A_0 \times 100\%$ (A and A_0 are the absorbance of experimental group and control group, respectively).

Cell culture. HeLa cells were cultured in DMEM supplemented with 1% penicillin/streptomycin and 10 % fetal bovine serum (FBS), and incubated in an atmosphere of 5/95 (v/v) of CO_2/air at 37 °C. Two days before imaging, the cells were passed and plated into 24-hole well with cover glass. For labeling, the cells were washed three times with phosphate buffered saline and then incubated with 5 μM **2a** in PBS (containing 1% DMSO) for 30 min at 37 °C. For positive and negative groups, the cells were pretreated with 1 mM NMM and 0.5 mM Cys for 30 min at 37 °C before labeling.

Two-photon and one-photon fluorescence bioimaging. Two-photon fluorescence microscopy images of HeLa cells (without pretreatment, with pretreatment of adding Cys and NMM) labeled with 5 μM **2a** were obtained with spectral confocal and multiphoton microscopes (NOL-LSM 710) with a ×100 oil objective, numerical aperture (NA=1.5). The two-photon fluorescence microscopy images were obtained by exciting the probes with a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength 730 nm, and collected the emission from 400~500 nm range. As comparison, HeLa cells were observed under the one-photon fluorescence microscope (Nikon Eclipse Ti-S) under UV light with ×20 objective.

Calculation of mean fluorescence intensity. The mean fluorescence density was measured by Image-Pro Plus (v. 6.0) and calculated via the equation (mean density= $IOD_{sum}/area_{sum}$), where IOD and area were integral optical density and area of fluorescent region.

Table S1. Absorption and emission maxima of **1a**~**1c** in DMSO.

Compound*	$\lambda_{max}^{(1)}$, nm	λ_{max}^{fl}, nm
1a	320	463
1b	336	451
1c	360	448

*: Compound **1a** was purchased from Sigma-Aldrich without further purification.

Table S2. Absorption and emission maxima of **1c** in various solvents.

Solvent (E_N^T)*	$\lambda_{max}^{(1)}$, nm	λ_{max}^{fl}, nm
1,4-dioxane (0.164)	357	453
DMSO (0.410)	360	448
EtOH (0.654)	354	428
H_2O (1.000)	364	408

*: The numbers in the parenthesis are normalized empirical parameter of solvent polarity.[7]

Table S3. Calculated frontier orbital energies (eV) of **1c**, **2a**, and **2b** at B3LYP/def2-SVP level.

	1c	**2a**	**2b**
LUMO+1	0.07268	-2.81572	-0.84243
LUMO	-1.05046	-3.33594	-1.17764
HOMO	-5.10772	-5.50567	-5.26124
HOMO-1	-5.92804	-6.38682	-6.0546

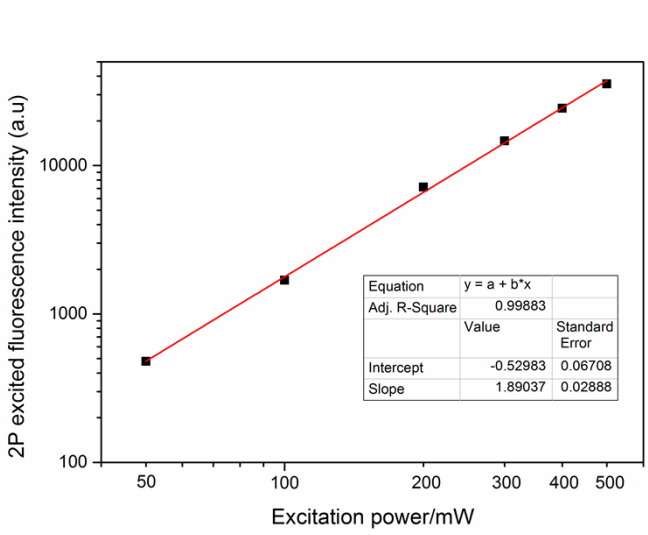

Fig. S1. The logarithmic relationship between 2P fluorescence intensity of **1c** and excitation power. The concentration of **1c** was 1 μM and the excitation wavelength was 730nm.

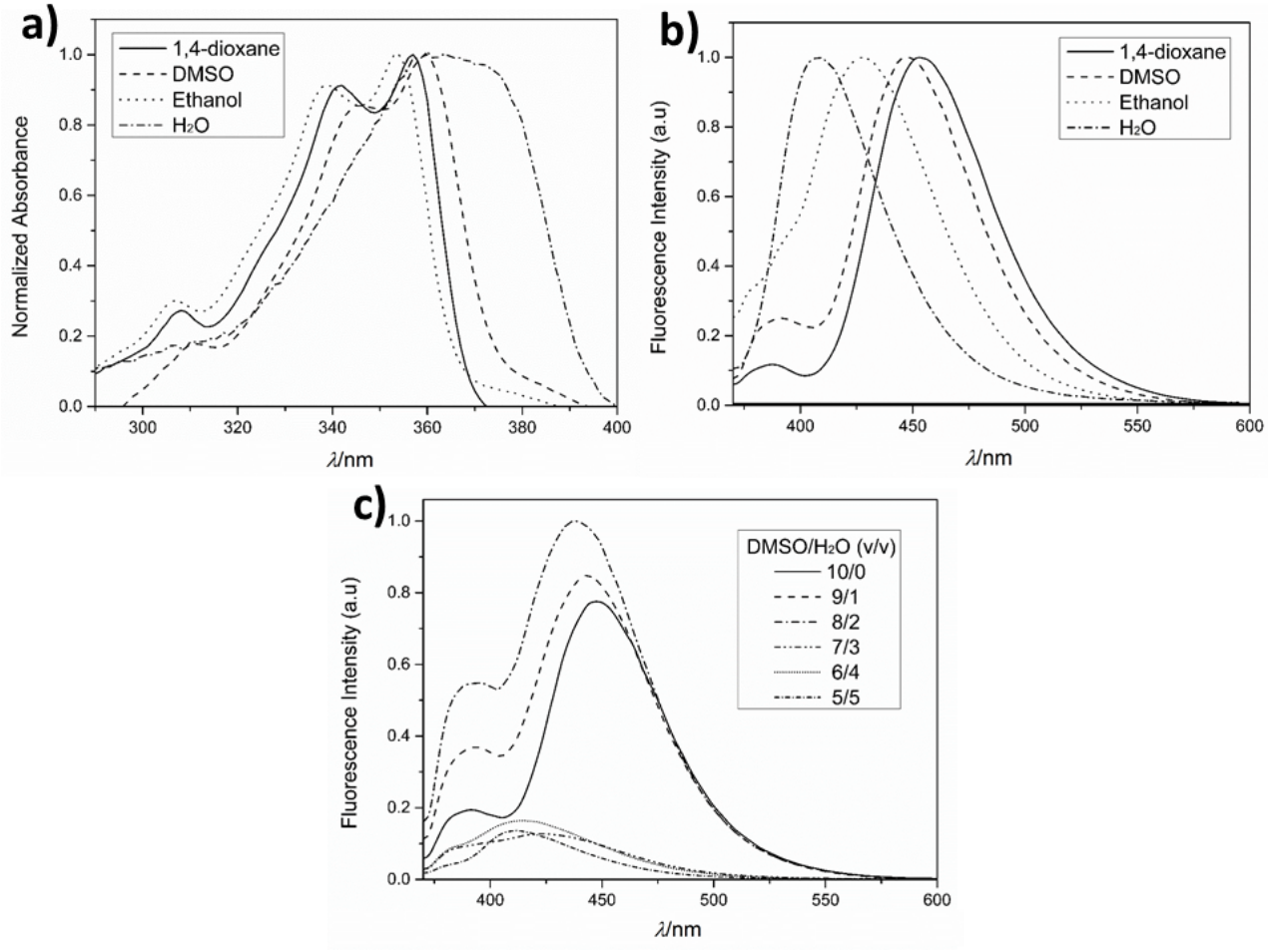

Fig. S2. Normalized absorption a) and emission b) spectra of **1c** in various solvents; c)Normalized fluorescence spectra of **1c** in DMSO/H_2O component solvent with different volume ratio.

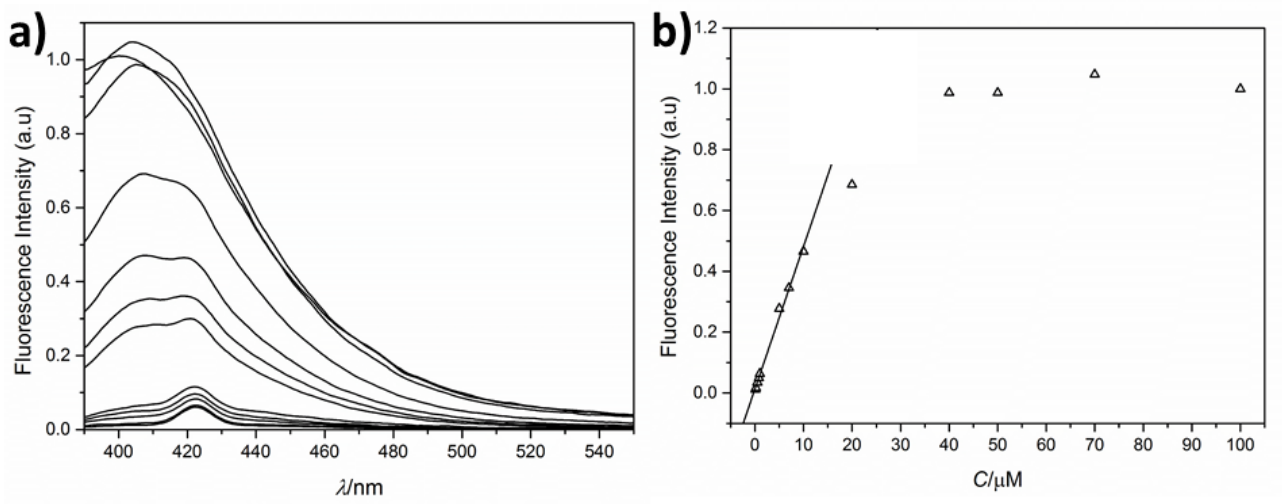

Fig. S3. One-photon fluorescence spectra a) and plots of fluorescence intensity against **2a** concentration b) in HEPES buffer (10 mM, pH 7.4) containing 0.2% DMSO. The excitation wavelength was 364 nm.

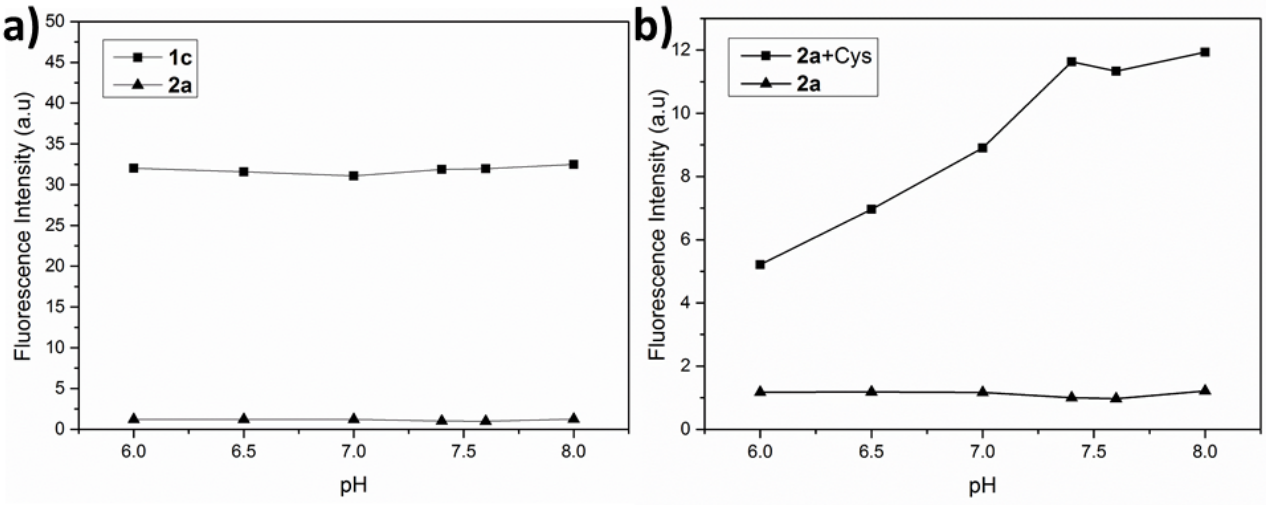

Fig. S4. a) Effect of pH on the one-photon fluorescence intensity of **2a** (▲) and **1c** (■); b) The fluorescence intensity of the reaction product between **2a** and Cys (●) compared to **2a** (▲) under different pH. Above detection were performed in DMSO/HEPES (8/2, V/V, 10 mM, different pH value) and spectra were collected under 360 nm excitation light.

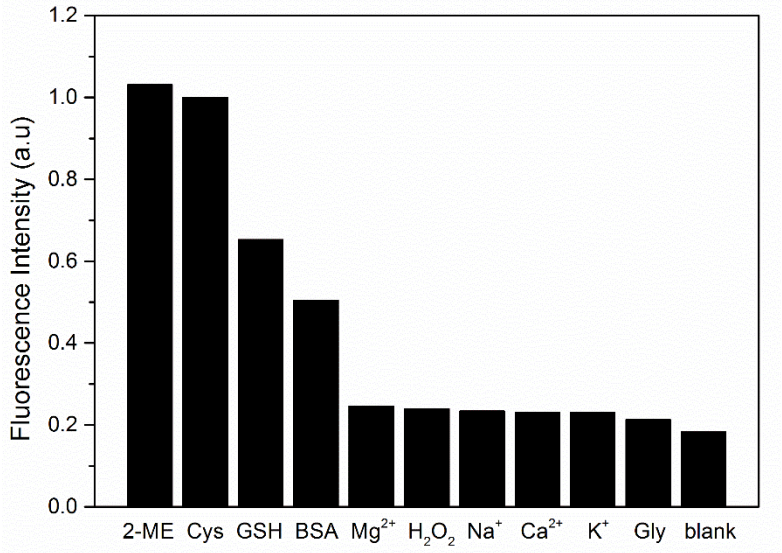

Fig. S5. The selectivity of probe **2a** towards thiol compounds and thiol-free substances in biological system. 2-ME (2-mercaptoethanol), Cys, GSH (glutathione), BSA (bull serum albumin): 10 μM; Mg^{2+}, H_2O_2, Na^+, Ca^{2+}, K^+, Gly (glycine): 1 mM. Above species were added into 10 μM **2a** in DMSO/HEPES (8/2, V/V, 10 mM, pH 7.4) component solvent. All spectra were collected under 360 nm excitation.

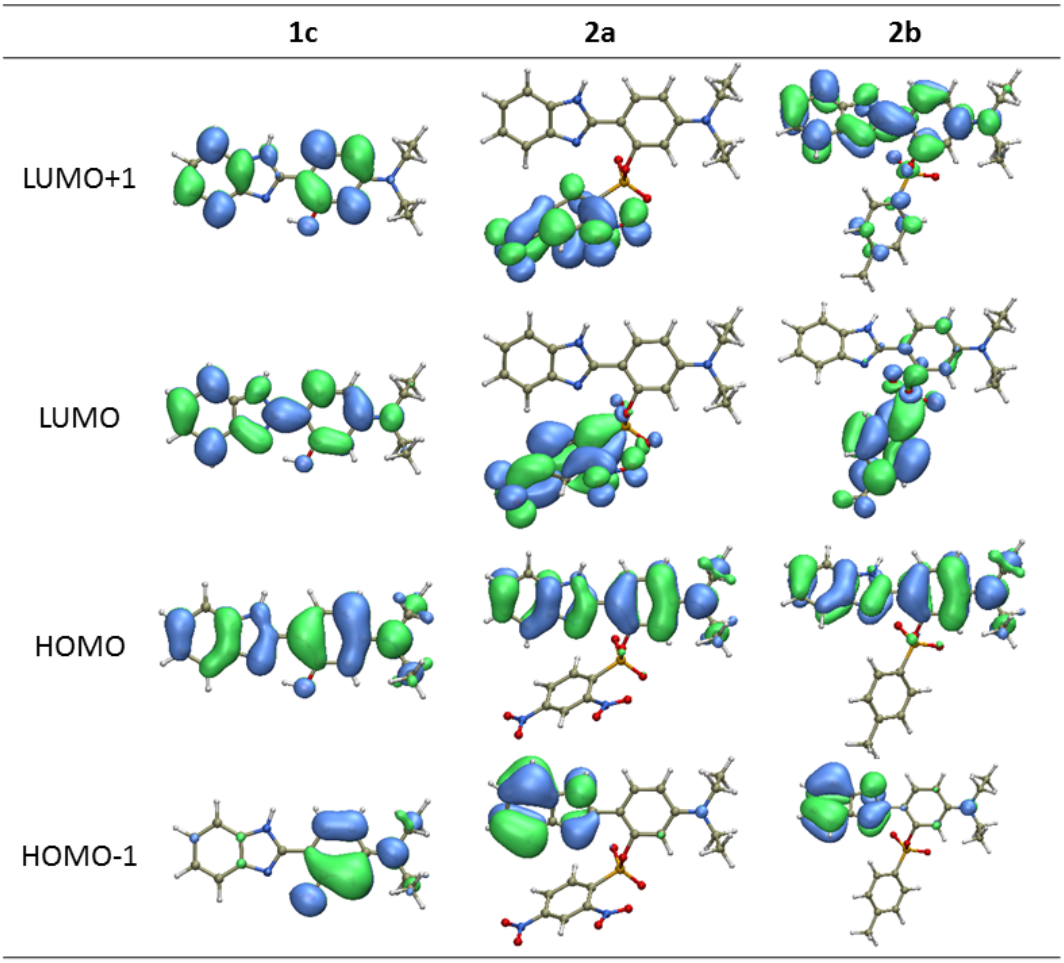

Fig. S6. The geometries and distribution of frontier orbitals in **1c**, **2a**, and **2b** at B3LYP/def2-SVP level.

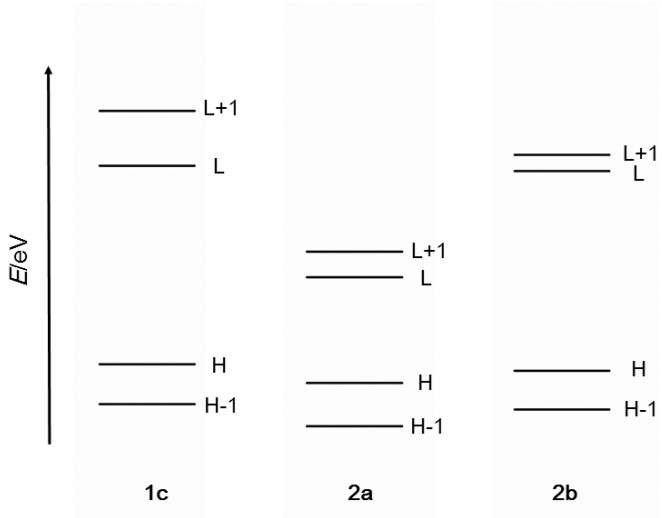

Fig. S7. Energy diagram for caculated frontier orbital energies of **1c**, **2a**, and **2b** at B3LYP/def2-SVP level.

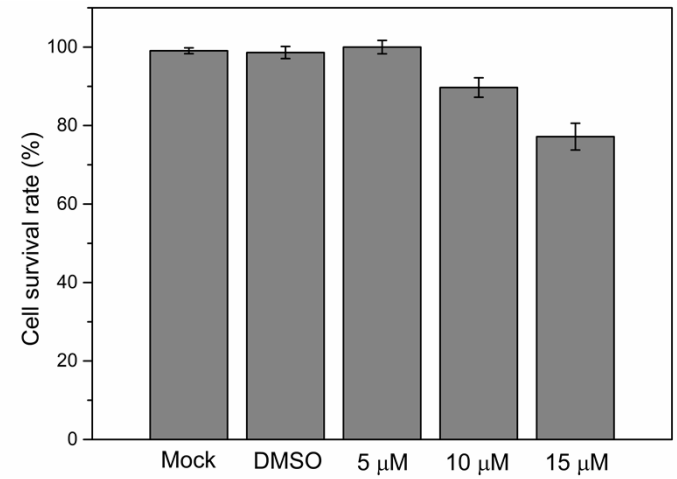

Fig. S8. Cell survival rate of control groups (without and with 1% DMSO in DMEM, represented by Mock and DMSO, respectively) and experimental group (with 5, 10, and 15 μM **2a**, containing 1% DMSO).

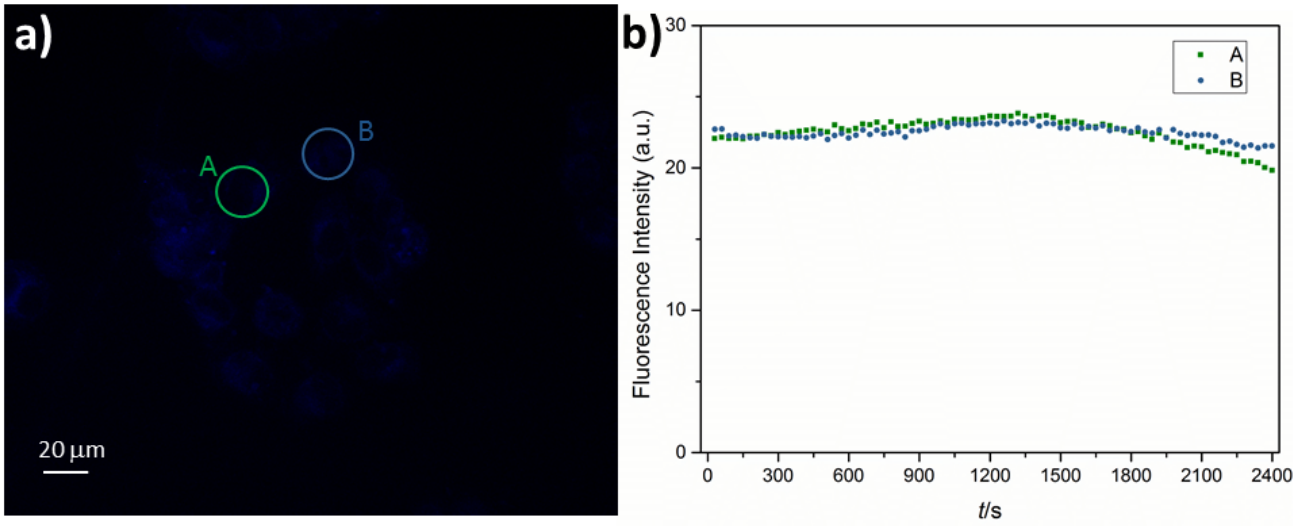

Fig. S9. a) 2PM images of HeLa cells labeled with 5 μM **2a**. b) 2P fluorescence intensity from circle A and B as a function of time. The 2P fluorescence intensity was collected with 30 sec intervals for the duration of 40 min under xyt mode. Images were collected at 400~500 nm upon excitation at 730 nm with femtosecond pulses (excitation power: 35 mW). Scale bar: 20 μm

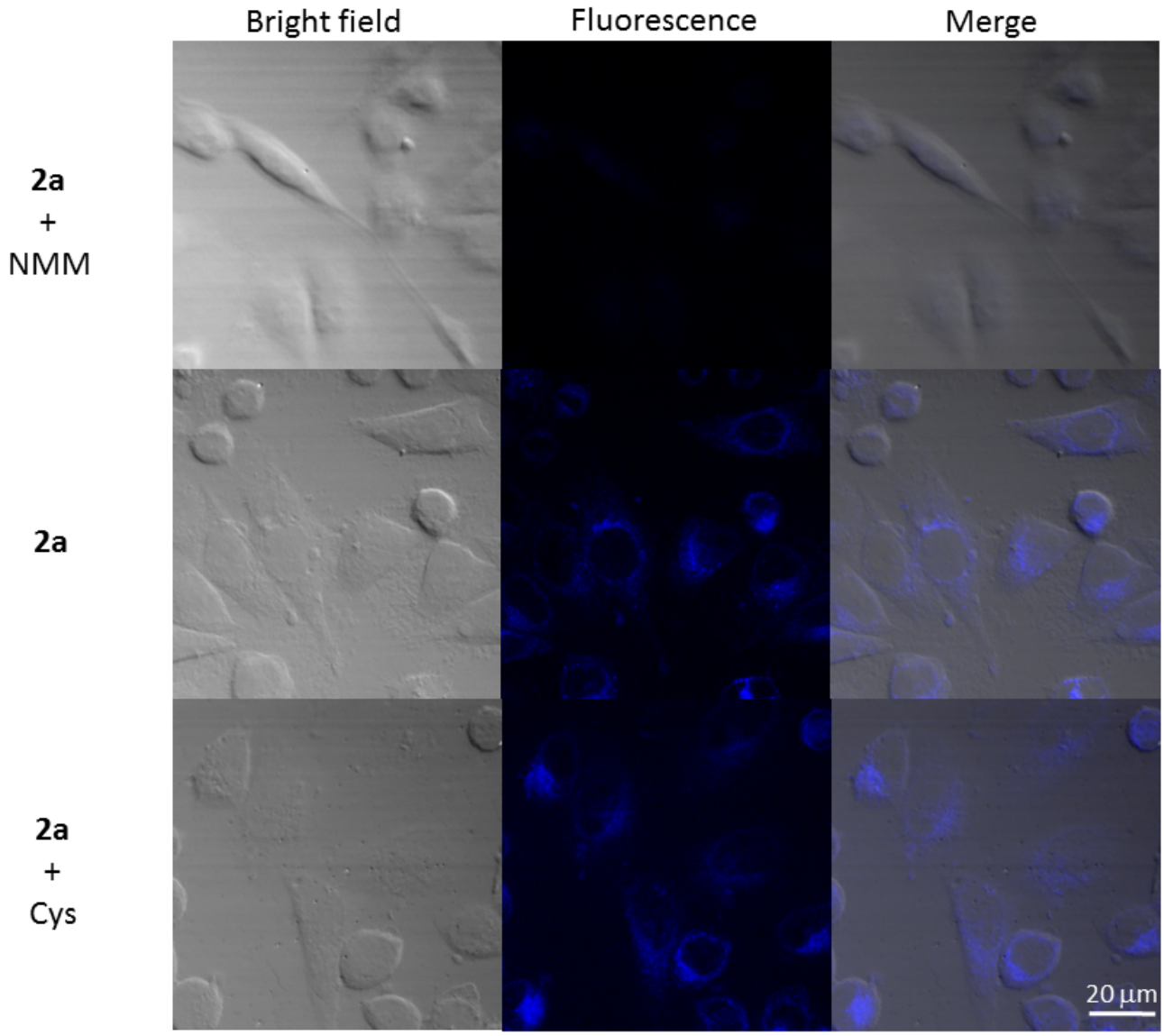

Fig. S10. Bright field, fluorescence and merged figures of the negative control group (5 μM **2a** + 1 mM NMM), experimental group (5 μM **2a**), and positive control group (5 μM **2a** + 0.5 mM Cys).

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