Electronic Supplementary Material (ESI) for Analyst.

Supporting information

Styrylpyridine salts-based far-red emissive two-photon turn-on probe for imaging the plasma membrane in living cells and tissues

Materials

Triphenylamine was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Phosphorus oxychloride was purchased from J&K Chemical (Beijing, China). 1-Iodododecane and Piperidine were from Beijing InnoChem Science & Technology Co., Ltd. The solvents used in the spectral measurement are of chromatographic grade.

Spectroscopic Measurements

The UV-visible-near-IR absorption spectra of dilute solutions were recorded on a U2910 spectrophotometer using a quartz cuvette owning 1 cm path length. Single-photon fluorescence spectra were obtained on a HITACH F-2700 spectrofluorimeter equipped with a 450-W Xe lamp. Two-photon fluorescence spectra were measured on a SpectroPro300i and the pump laser beam came from a mode-locked Ti: sapphire laser system at the pulse duration of 220 fs, a repetition rate of 76 MHz (Coherent Mira900-D). All pH measurements were performed with a METTLER TOLEDO FE20-FiveEasy^{TM} pH-meter.

The fluorescence quantum yields can be calculated by the following equation (1):

$$\Phi_s = \Phi_r \left(\frac{A_r(\lambda_r)}{A_s(\lambda_s)} \right) \frac{c_r}{c_s} \frac{n_s^2}{n_r^2} \frac{F_s}{F_r} \qquad (1)$$

where the subscripts *s* and *r* refer to the sample and the reference materials, respectively. *Φ* is the quantum yield, *F* is the integrated emission intensity, *A* stands for the absorbance, and *n* is the refractive index. In this paper, the quantum yields were calculated by using fluorescein in aqueous NaOH (pH = 13, Φ = 0.93) [1] as a

standard.

Two-photon absorption cross sections have been measured using the two-photon induced fluorescence method. The δ of fluorescein in aqueous NaOH under different wavelengths was used as the standard, whose two-photon properties have been characterized in the literature, and thus cross sections can be calculated by means of equation (2): [2-4]

$$\delta_s = \delta_r \frac{\Phi_r}{\Phi_s} \frac{c_r}{c_s} \frac{n_r}{n_s} \frac{F_s}{F_r} \qquad (2)$$

where the subscripts *s* and *r* refer to the sample and the reference materials, respectively. *δ* is the two-photon absorption cross-section value, *c* is the concentration of the solution, *n* is the refractive index of the solution, *F* is the two-photon excited fluorescence integral intensity and *Φ* is the fluorescence quantum yield. The *δ* of fluorescein at different excitation wavelengths were calculated by using fluorescein in aqueous NaOH (pH = 13, Φ = 0.93) [5] as a standard.

Cell culture, cell and tissue staining

HeLa cells were cultured in H-DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin in a 5% CO_2 incubator at 37 ^oC. HUVEC cells were grown in M199 medium supplemented with 10% FBS and 2 ng/mL FGF-2. **TSP** was dissolved in DMSO at a concentration of 0.1 mM and S-11348 and Hoechst 33342 were prepared as 5 mM in DMSO.

For living cells staining experiments, cultured cells grown on glass coverslips were stained with 2 μM **TSP** in culture medium for 20 min at 37 ^oC and then imaged with fluorescence microscopy.

For cells counterstain experiment with S-11348 or Hoechst 33342 : live cells were incubated with 2 μM **TSP** in culture medium for 20 min at 37 ^oC, then stained with S-11348 (5 μM, 5 min) or Hoechst 33342 (5 μM, 30 min). After that, the sample was rinsed with D-Hanks twice.

Cell-viability assay: the study of the effect of **TSP** on viability of cells was carried out using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. HeLa cells

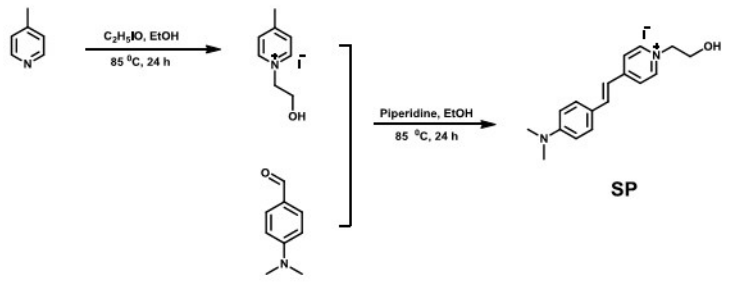
growing in log phase were seeded into 96-well plates (ca. 1 × 10^4 cells/well) and allowed to adhere for 24 h. **TSP** (200 μL/well) at concentration of 2 μM was added into the wells of the treatment group, and 200 μL/well DMSO diluted in DMEM at final concentration of 0.2% to the negative control group, respectively. The cells were incubated for 2, 9, and 24 h at 37 °C under 5% CO_2, then MTT (5 mg/mL in phosphate buffer solution) was added into each well. After 4 h incubation at 37 °C, 200 μL DMSO was added to dissolve the purple crystals. After 20 min incubation, the optical density readings at 570 nm were taken using a plate reader. Cytotoxic experiment was repeated for four times.

Tissue staining: the rat muscular and hepatic tissues were directly removed from just killed adult wistar rat, and then stained with **TSP** (10 μM, 40 min) at room temperature in H-DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin.

Fluorescence imaging

Two-photon fluorescence imaging was obtained with IX71 (Olympus) multiphoton laser scanning microscope with Coherent Mira900-D. Confocal fluorescence imaging was obtained with LSM 780 (Zeiss) confocal laser scanning microscope or Olympus FV 300 laser confocal microscope. The DIC images were taken with 488 nm Ar^+ ion laser.

Synthesis and characterization



Scheme S1: The synthesis routine of **SP**.

(E)-4-(4-(dimethylamino)styryl)-1-(2-hydroxyethyl)pyridin-1-ium iodide

(SP)

The mixture comprised of 4-methylpyridine (2 mL, 20 mmol) and 2-iodoethanol (1.56 mL, 20 mmol) was added into a flask, with ethyl alcohol (5 mL) as the solvent. Next, the reaction system was stirred for 24 h at 85 °C. After that, adding 4-(dimethylamino)benzaldehyde (2.98 g, 20 mmol) and 200uL piperidine into this mixture with stirring at 85 °C for 24 h. After being cooled to room temperature, the precipitate was washed with little ethyl alcohol two times and then petroleum ether three times. Red power product was obtained after the residue was recrystallized from ethyl alcohol, with a yield of 80%. 1H NMR (400 MHz, DMSO-d6): δ (ppm) 8.70 (d, *J* = 6.8 Hz, 2H), 8.07 (d, *J* = 6.8 Hz, 2H), 7.92 (d, *J* = 16 Hz, 1H), 7.60 (d, *J* =8.8, 1H), 7.19 (d, *J* = 16.4 Hz, 1H), 6.79 (d, *J* = 8.8, 2H), 5.21 (t, *J* = 5.2 Hz, 1H), 4.47 (t, *J* = 5.0 Hz, 2H).

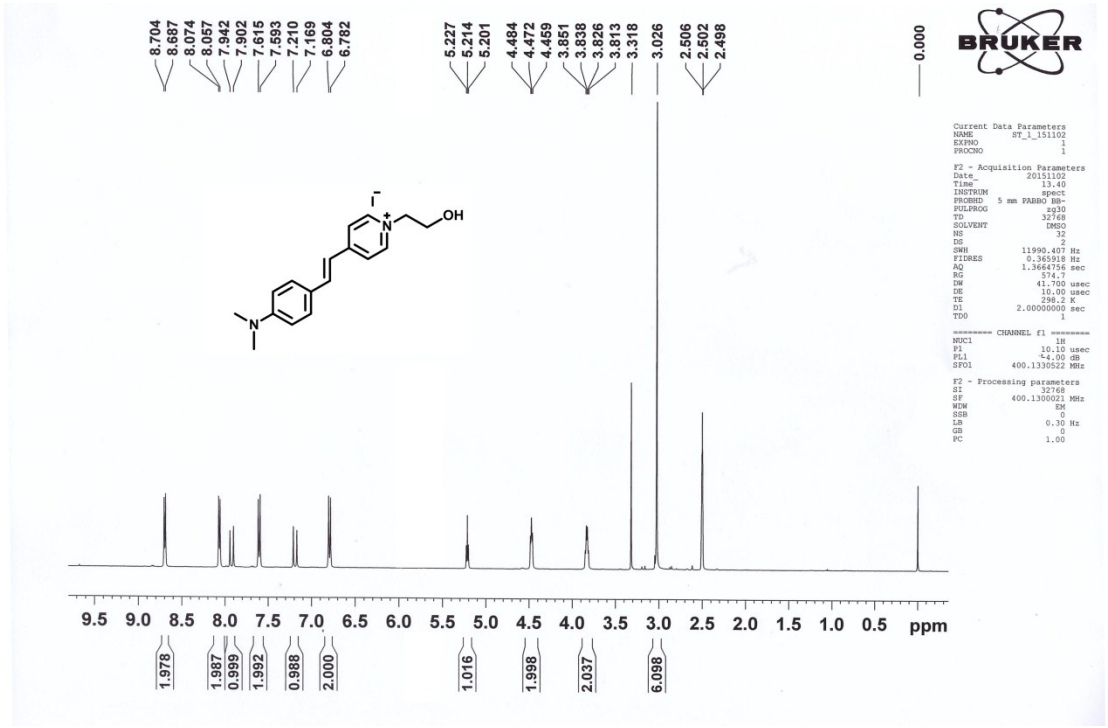


Fig. S1 ^{1}H NMR spectrum of **SP** in DMSO-*d6*.

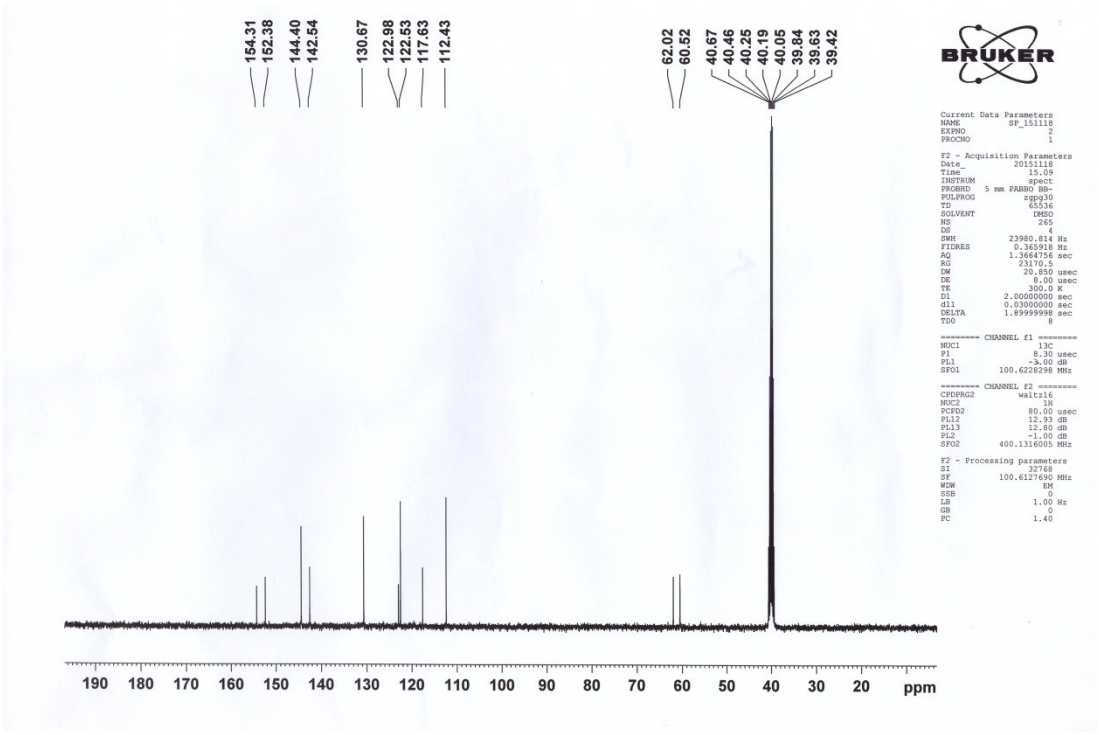


Fig. S2 ^{13}C NMR spectrum of **SP** in DMSO-*d6*.

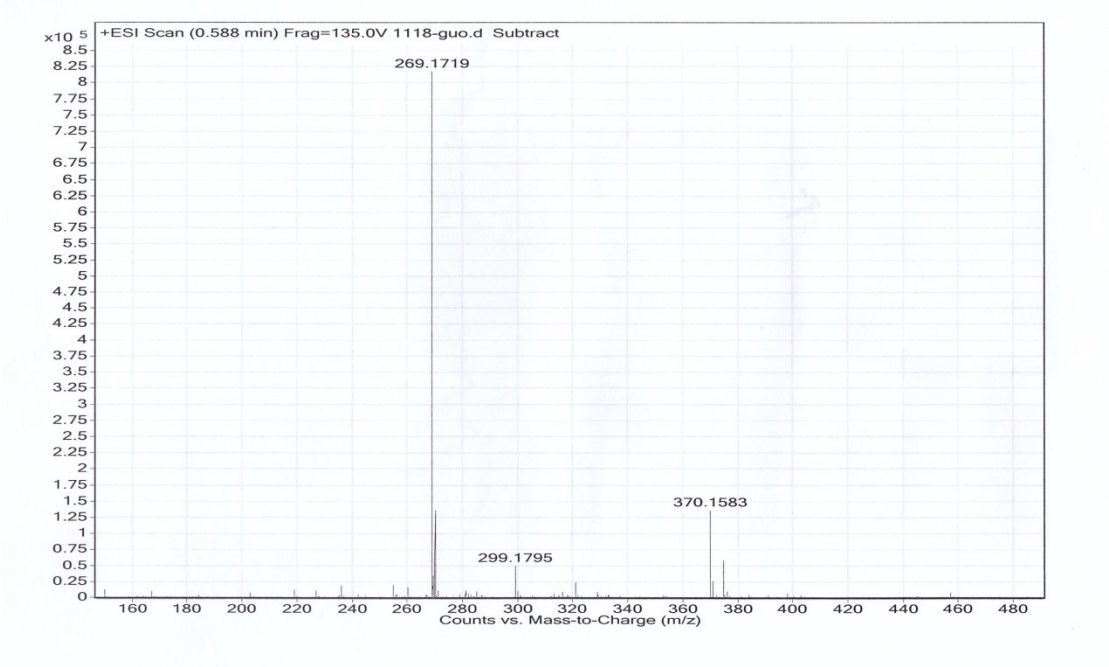


Fig. S3 HRMS spectrum of **SP** in MeOH.

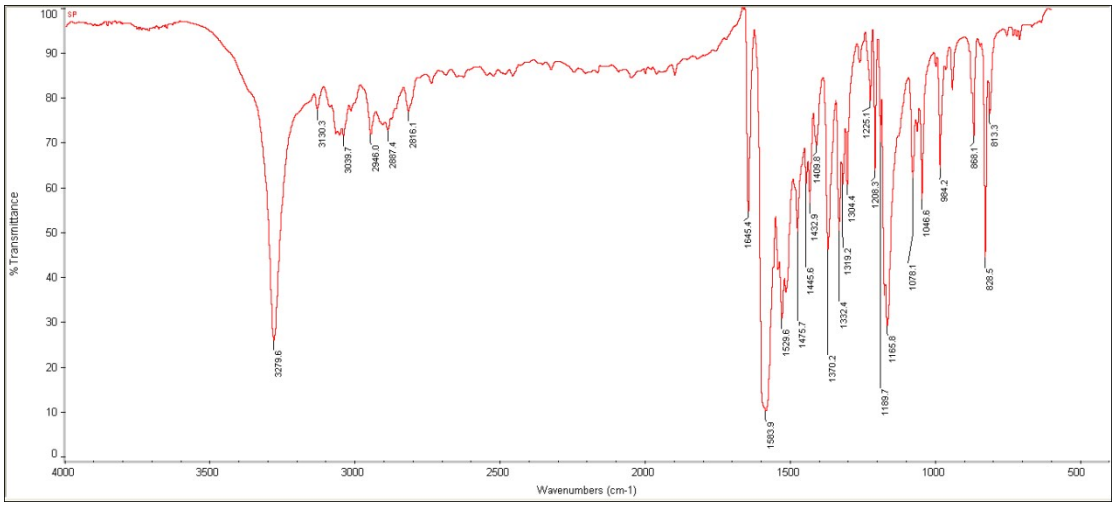
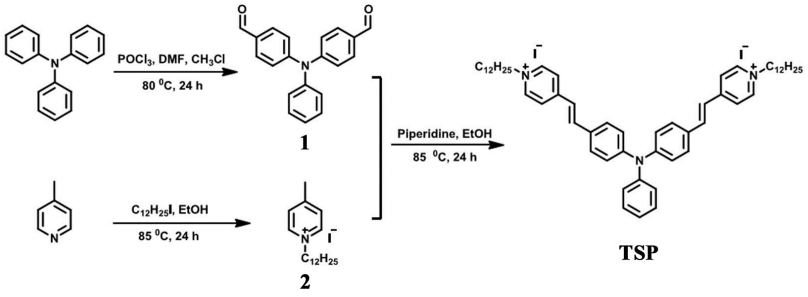


Fig. S4 IR spectrum of **SP**.



Scheme S2: The synthesis routine of **TSP**.

4,4'-(phenylazanediyl)dibenzaldehyde (1):

The phosphoryl chloride (3.7 mL, 40.7 mmol) was added to the dryed dimethylformamide (3.7 mL) at 0 °C with stirring in a flask. After 30 min, triphenylamine (1.0 g, 4.0 mmol) dissolved with CH_3Cl was added into this system for 1 h, and then the mixture was warmed at 80 °C for 24 h. After being cooled to room temperature, the mixture was poured into sodium hydroxide and 200 mL water, and extracted with dichloromethane. After that, the organic compound was washed two times, and dried with anhydrous magnesium sulfate for 3 h. Finally, the pured products were obtained by column chromatography with ethyl acetate/petroleum ether (1:8, v/v) as eluent, and the yield is 40%. ^{1}H NMR (400 MHz, DMSO-*d6*): δ (ppm) 9.88 (s, 2H), 7.85 (d, *J* = 8.64 Hz, 4H), 7.49 (t, *J* = 7.84 Hz, 2H), 7.33 (t, *J* = 7.4 Hz, 1H), 7.22 (d, *J* = 8.4 Hz, 2H), 7.17 (d, *J* = 8.56 Hz, 4H).

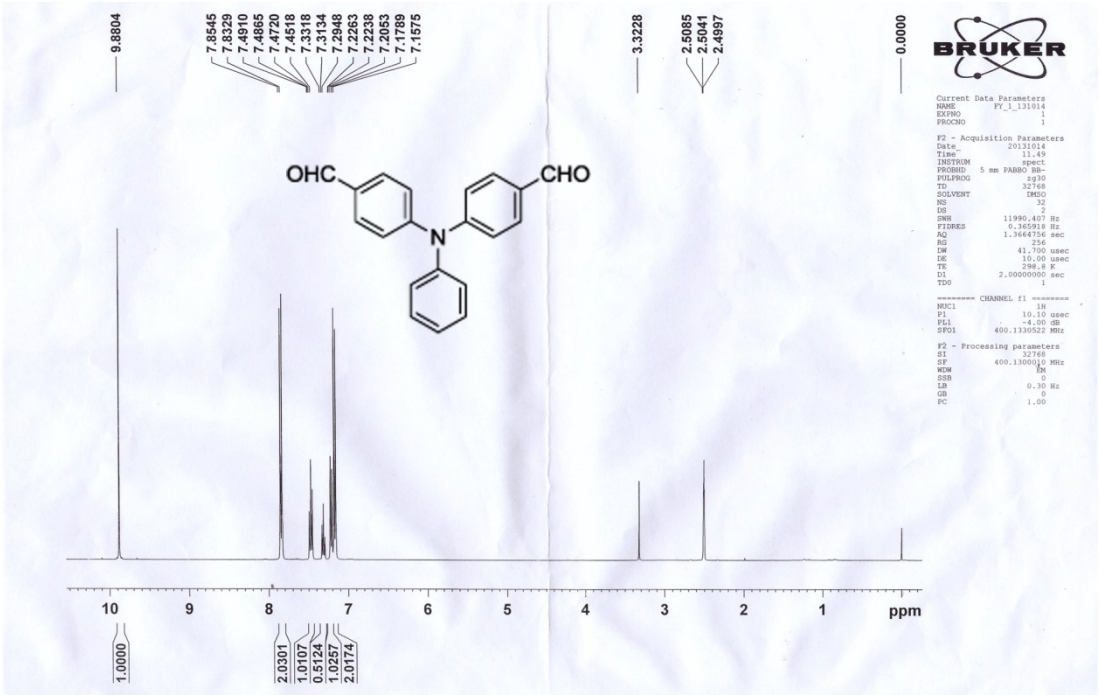


Fig. S5 ^{1}H NMR spectrum of (1) in DMSO-*d6*.

1-dodecyl-4-methylpyridin-1-ium iodide (2):

The mixture comprised of 4-methylpyridine (2 mL, 20 mmol) and 1 - iodine dodecane (6 mL, 24 mmol) was added into a flask, with ethyl alcohol (5 mL) as the solvent. Next, the reaction system was stirred for 24 h at 85 ^{o}C. After that, the resulting mixture was distilled to remove ethyl alcohol, and then the crude product was acquired. Further, after being washed with petroleum ether three times, the final products was obtained with the yield of 80.0%. ^{1}H NMR (400 MHz, DMSO-*d6*): δ (ppm) 8.93 (d, *J* = 6.4 Hz, 2H), 7.99 (d, *J* = 6.4 Hz, 2H), 4.52 (t, *J* = 7.4 Hz, 2H), 2.61 (s, 3H), 1.89 (q, *J* = 7.2 Hz, 2H), 1.23 (s, 18H), 0.85 (t, *J* = 6.8 Hz, 3H).

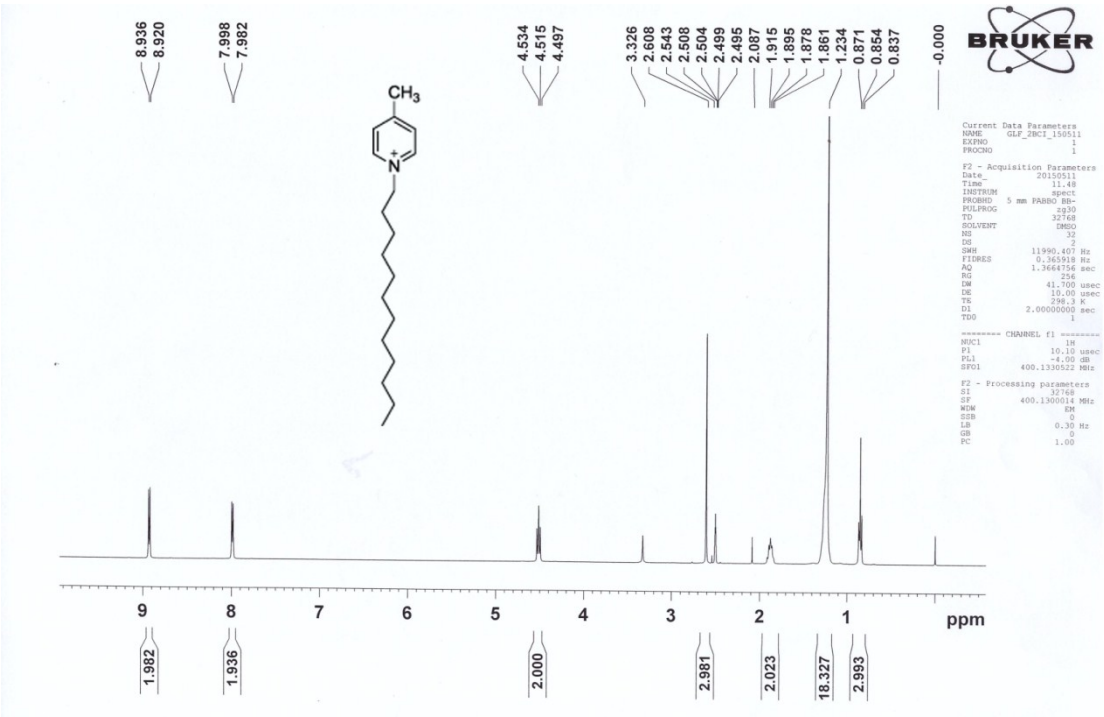


Fig. S6 ^{1}H NMR spectrum of (2) in DMSO-*d6*.

*4,4'-((1E,1'E)-((phenylazanediyl)bis(4,1-phenylene))bis(ethene-2,1-diyl))bis(1-dodecylpyridin-1-ium) iodide (**TSP**):*

Compound (1) (0.48 g, 1.6 mmol) and compound (2) (1.24 g, 3.2 mmol) was dissolved in 20 mL ethyl alcohol, and then added into a flask. Sequently, 200 uL piperidine as catalyst, was added into this mixture with stirring at 85 ^{o}C for 24 h. After being cooled to room temperature, the precipitate was washed with little ethyl alcohol two times and then petroleum ether three times. Red power product was obtained after the residue was recrystalled from ethyl alcohol, with a yield of 80%. ^{1}H NMR (400 MHz, DMSO-*d6*): δ (ppm) 8.91 (d, *J* = 6.8 Hz, 4H), 8.20 (d, *J* = 6.8 Hz, 4H), 8.01 (d, *J* = 16 Hz, 2H), 4.47 (t, *J* = 7.2 Hz, 4H), 1.91 (d, *J* = 6.4 Hz, 4H), 1.27 (d, *J* = 5.2 Hz, 8H), 1.24 (s, 28H), 0.85 (t, *J* = 6.8 Hz, 6H).

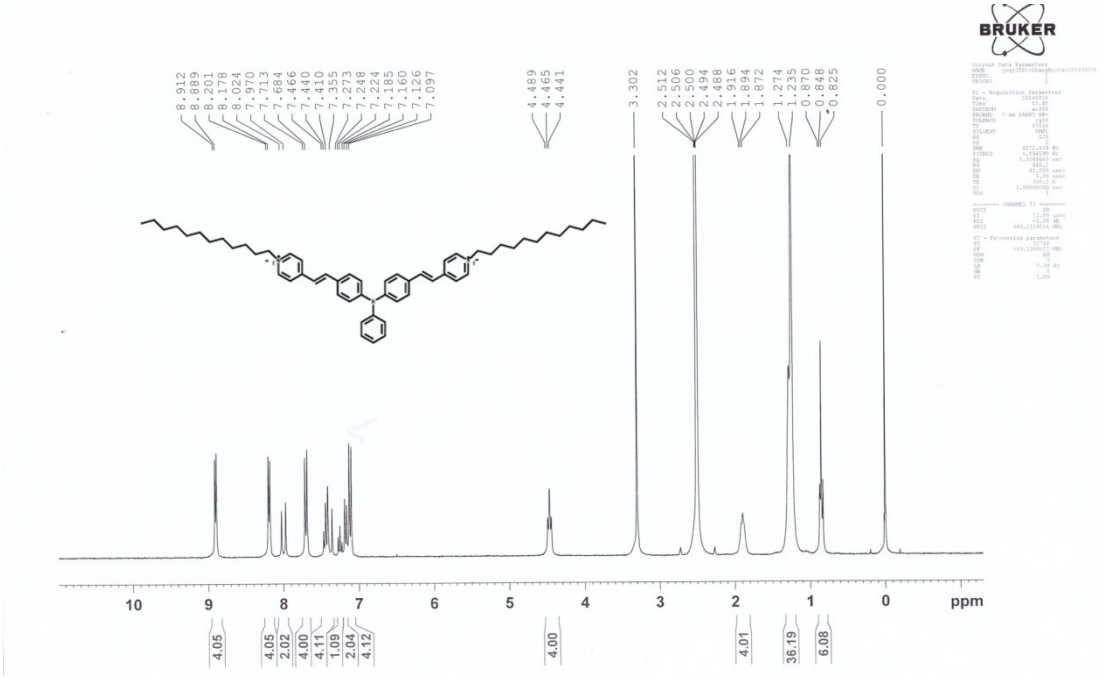


Fig. S7 ^{1}H NMR spectrum of **TSP** in DMSO-*d6*.

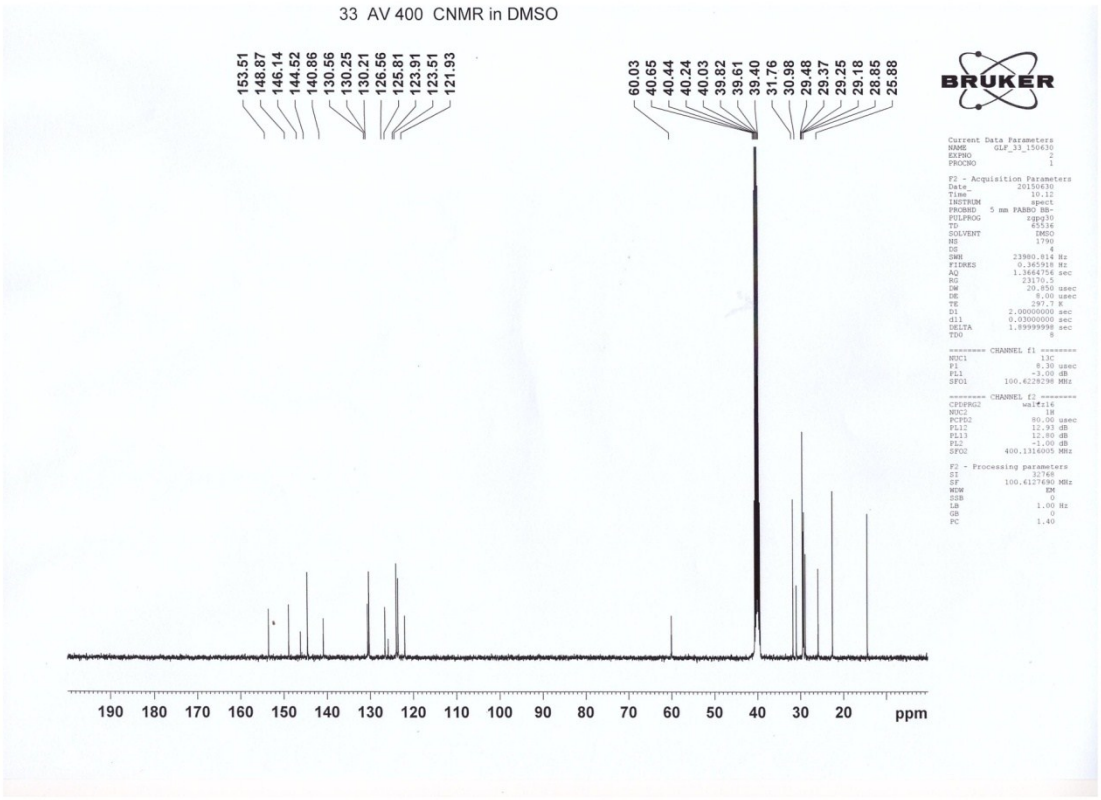


Fig. S8 ^{13}C NMR spectrum of **TSP** in DMSO-*d6*.

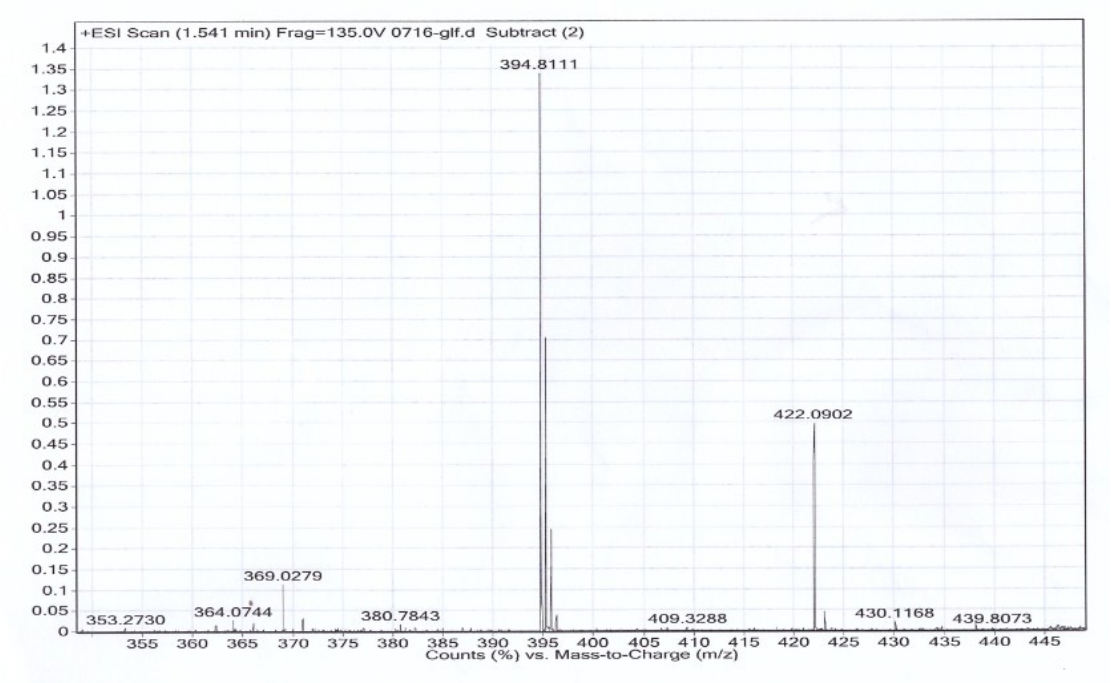


Fig. S9 HRMS spectrum of **TSP** in MeOH.

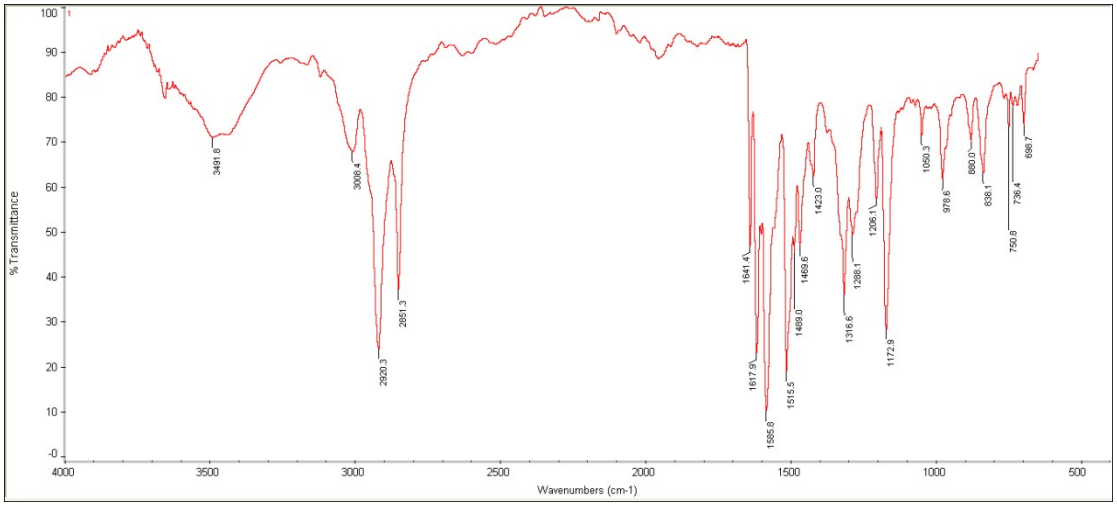


Fig. S10 IR spectrum of **TSP**.

Spectrum spectrogram and fluorescence images

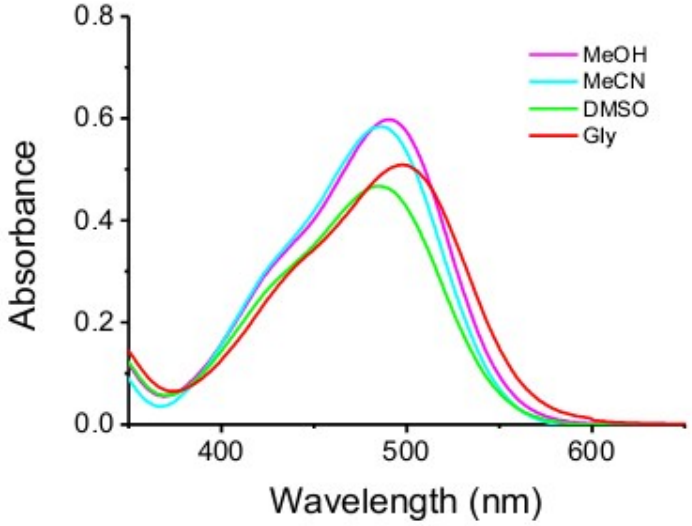


Fig S11. The absorption spectra of **TSP** (10 μM) in various solvents.

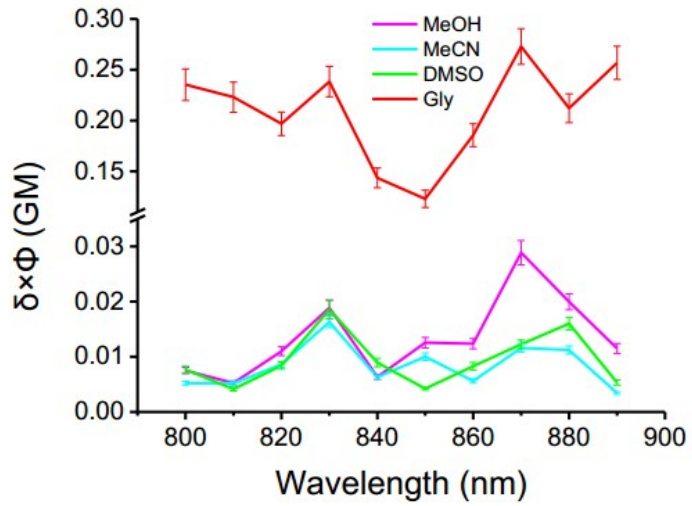


Fig S12. (δ × Φ) of **TSP** (2 μM) in different solvents excited by different excitation wavelengths.

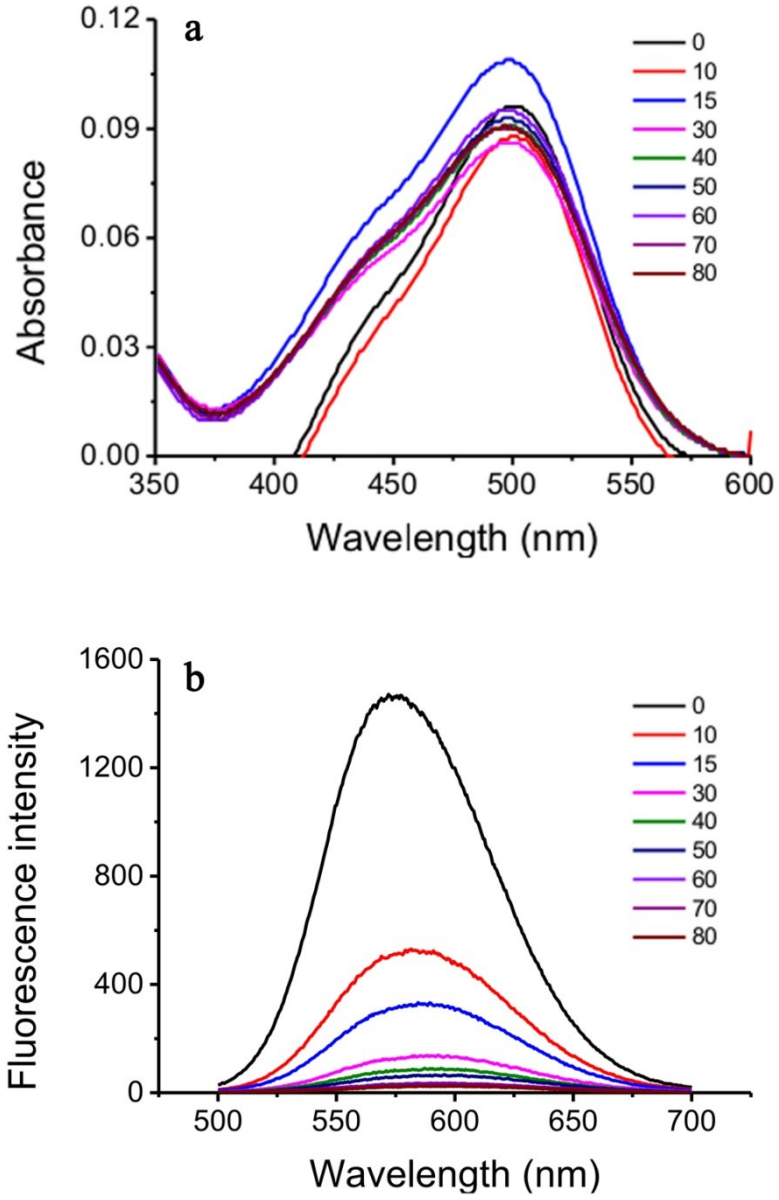


Fig S13. The absorption (a) and fluorescence (b) spectra of **TSP** (2 μM) in Gly at different temperatures. λ_{ex} = 488 nm.

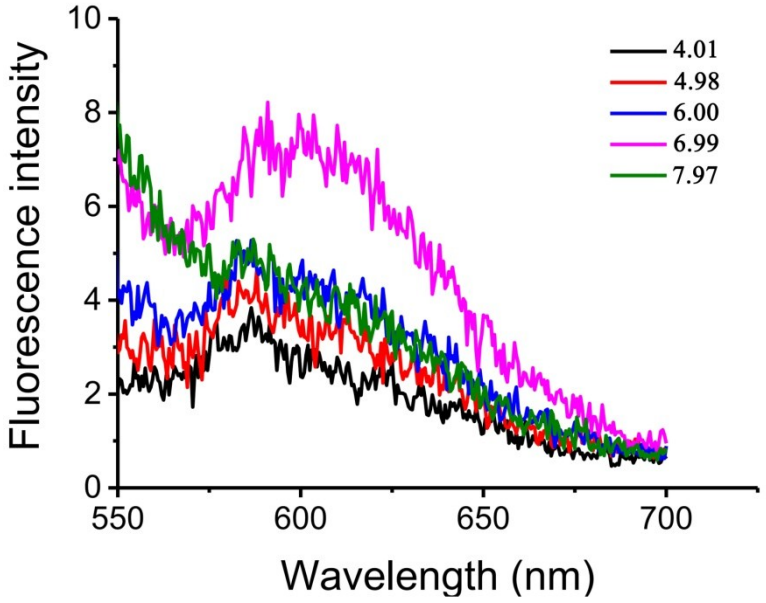


Fig S14. The fluorescence spectra of **TSP** (2 μM) in PBS buffer with different pH values. λ_{ex} = 488 nm.

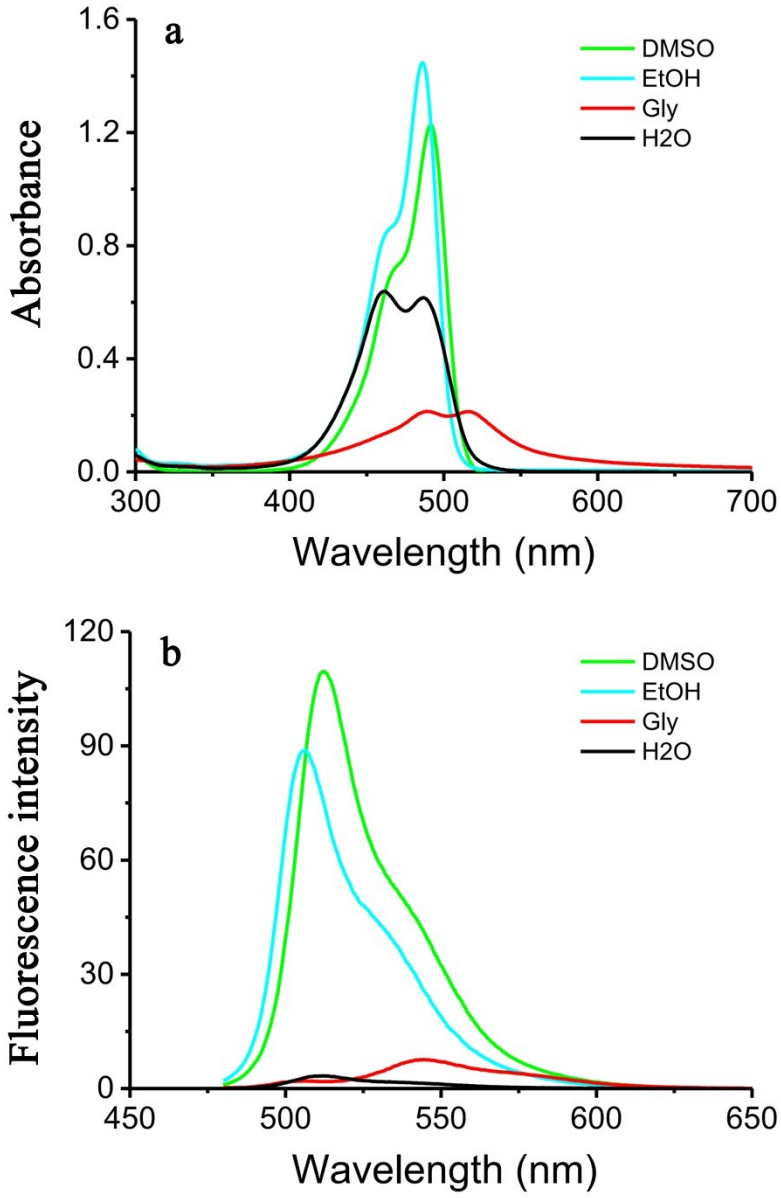


Fig S15. The absorption and fluorescence spectra of DiO (10 μM) in various solvents. λ_{ex} = 488 nm.

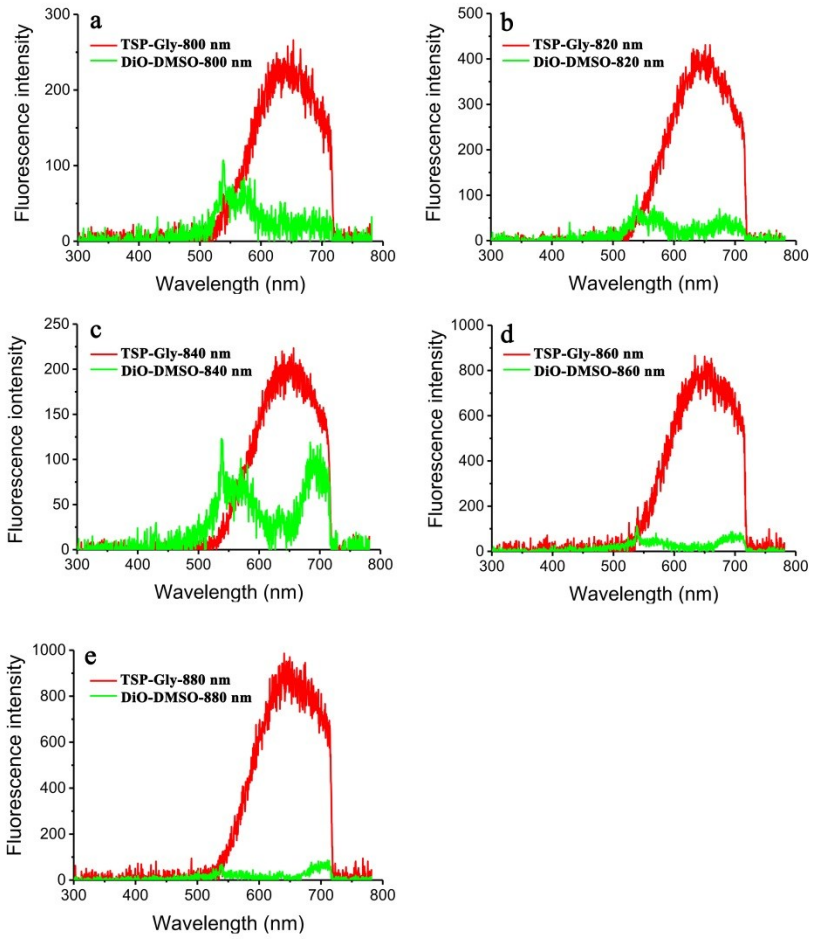


Fig S16. TPEF spectra of **TSP** (2 μM) in Gly and DiO (2 μM) in DMSO under different excitation wavelengths ranging from 800 to 880 nm.

Vedio S1: The vedio of TPEF images of HeLa cells stained with **TSP** (2 μM, 20 min) at different depths. Ex = 800 nm, Em < 720 nm. Bar = 20 μm.

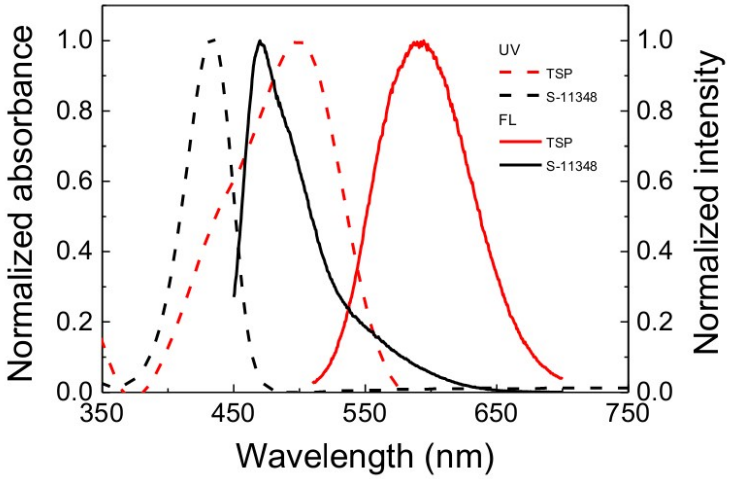


Fig S17. The absorption and fluorescence spectra of **TSP** (10 μM) in Gly and S-11348 (10 μM) in DMSO. λ_{ex} (**TSP**) = 488 nm, λ_{ex} (S-11348) = 405 nm.

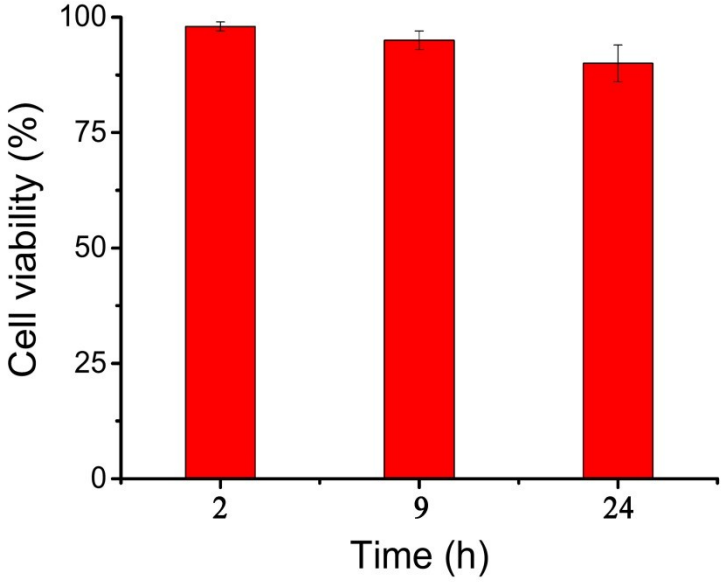


Fig S18. Cytotoxicity data of HeLa cells treated with **TSP** by MTT assay.

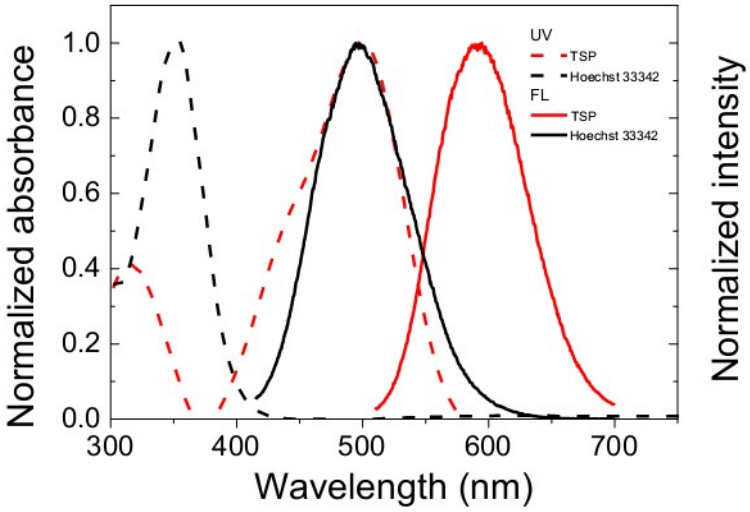


Fig S19. The absorption and fluorescence spectra of **TSP** (10 μM) in Gly and Hoechst 33342 (10 μM) in DMSO. λ_{ex} (**TSP**) = 488 nm, λ_{ex} (Hoechst 33342) = 405 nm.

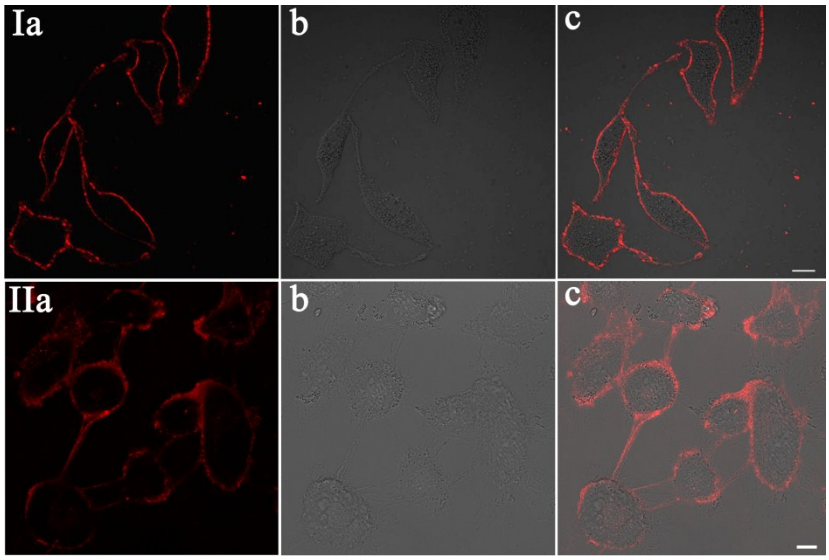


Fig S20. Confocal fluorescence images of SiHa cells (I) and HUEVC (II) stained with **TSP** (2 μM, 20 min). (a) Fluorescence images incubated with **TSP** (λ_{ex} = 488 nm, λ_{em} =550-700 nm); (b) DIC images; (c) Merged images.

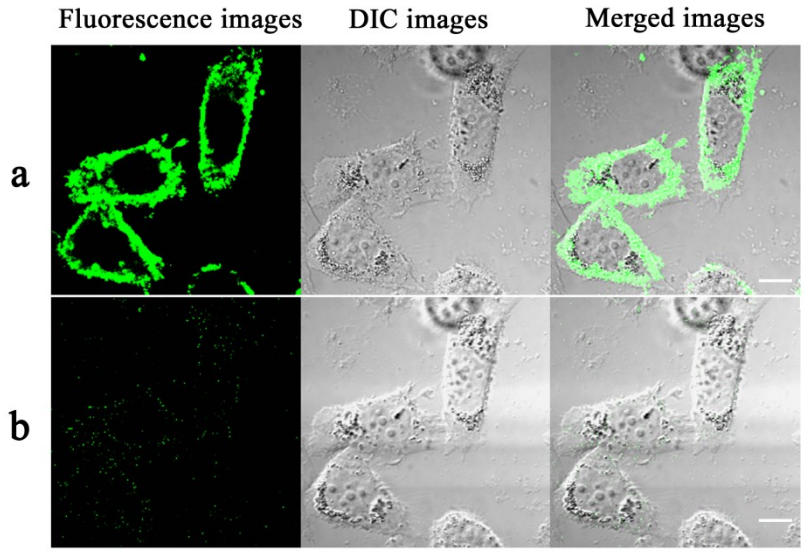


Fig S21. Images of HeLa cells stained with 5 μM DiO for 30 min at 37 °C. (a) SPEF images (λ_{ex} = 405 nm, λ_{em} = 510-540 nm); (b) TPEF images (λ_{ex} = 800 nm, λ_{em} < 720 nm). Bar = 20 μm.

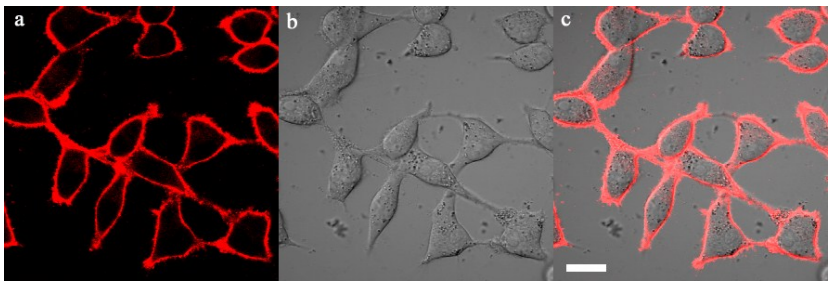
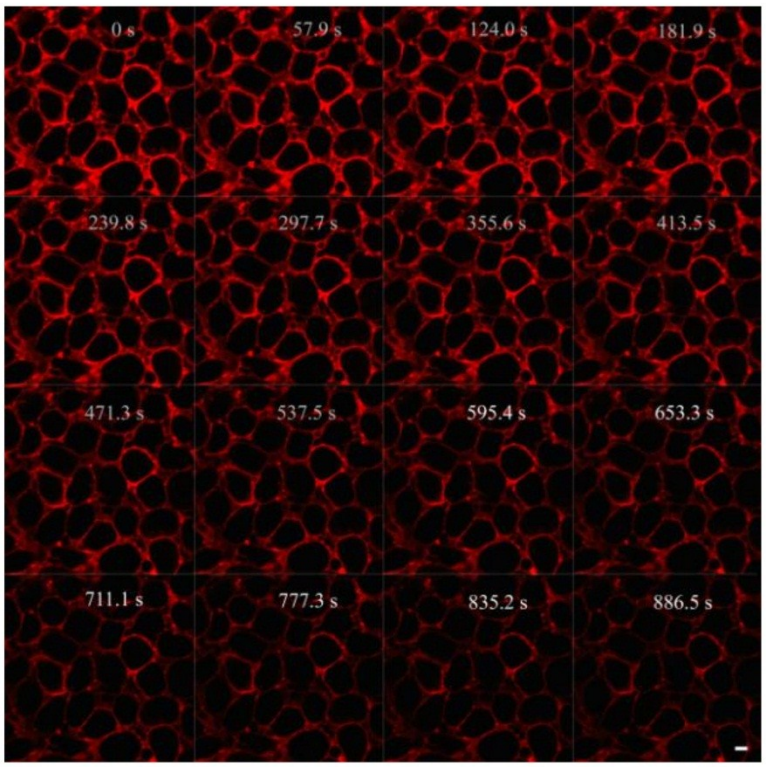


Fig S22. TPEF images of HeLa cells stained with 2 μM TSP for 20 min at 37 °C. (a) Fluorescence images; (b) DIC images; (c) Merged images. λex = 800 nm, λem < 720 nm.. Bar = 20 μm.

a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181.9 s
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537.5 s
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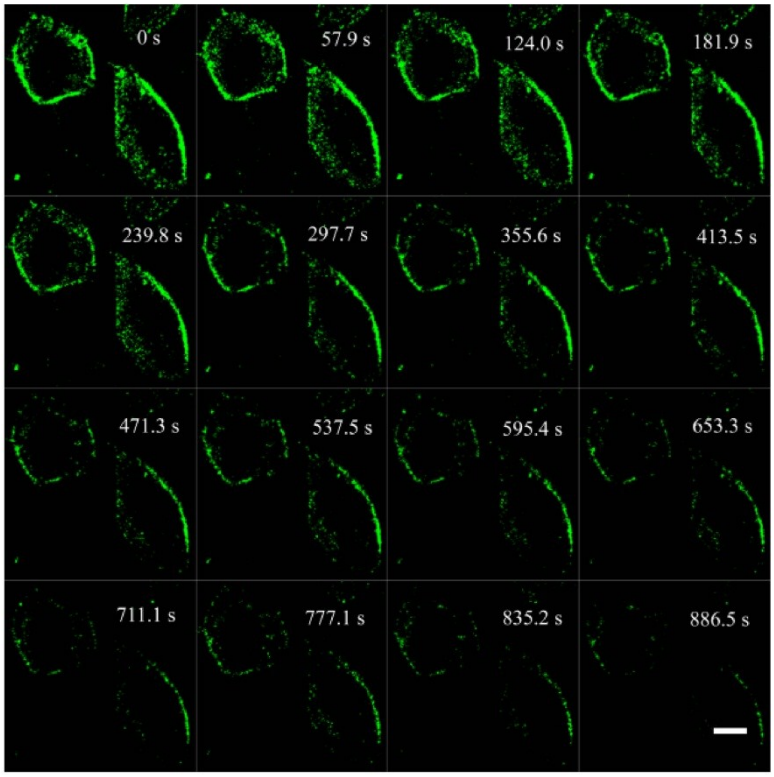
653.3 s
711.1 s
777.3 s
835.2 s
886.5 s

b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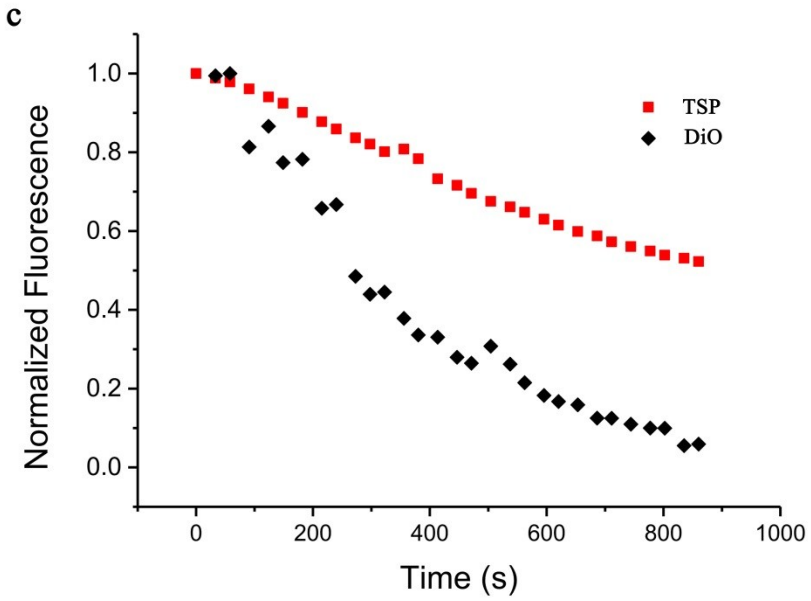

Fig S22. SPEF images of HeLa cells stained with **TSP** (a, 2 μM, 20 min) and DiO (b, 5 μM, 30 min) at different time under ceaseless exposure of the same laser source respectively, and the corresponding fluorescence intensity (c). λ_{ex} (**TSP**, DiO) = 488 nm. Bar = 20 μm.

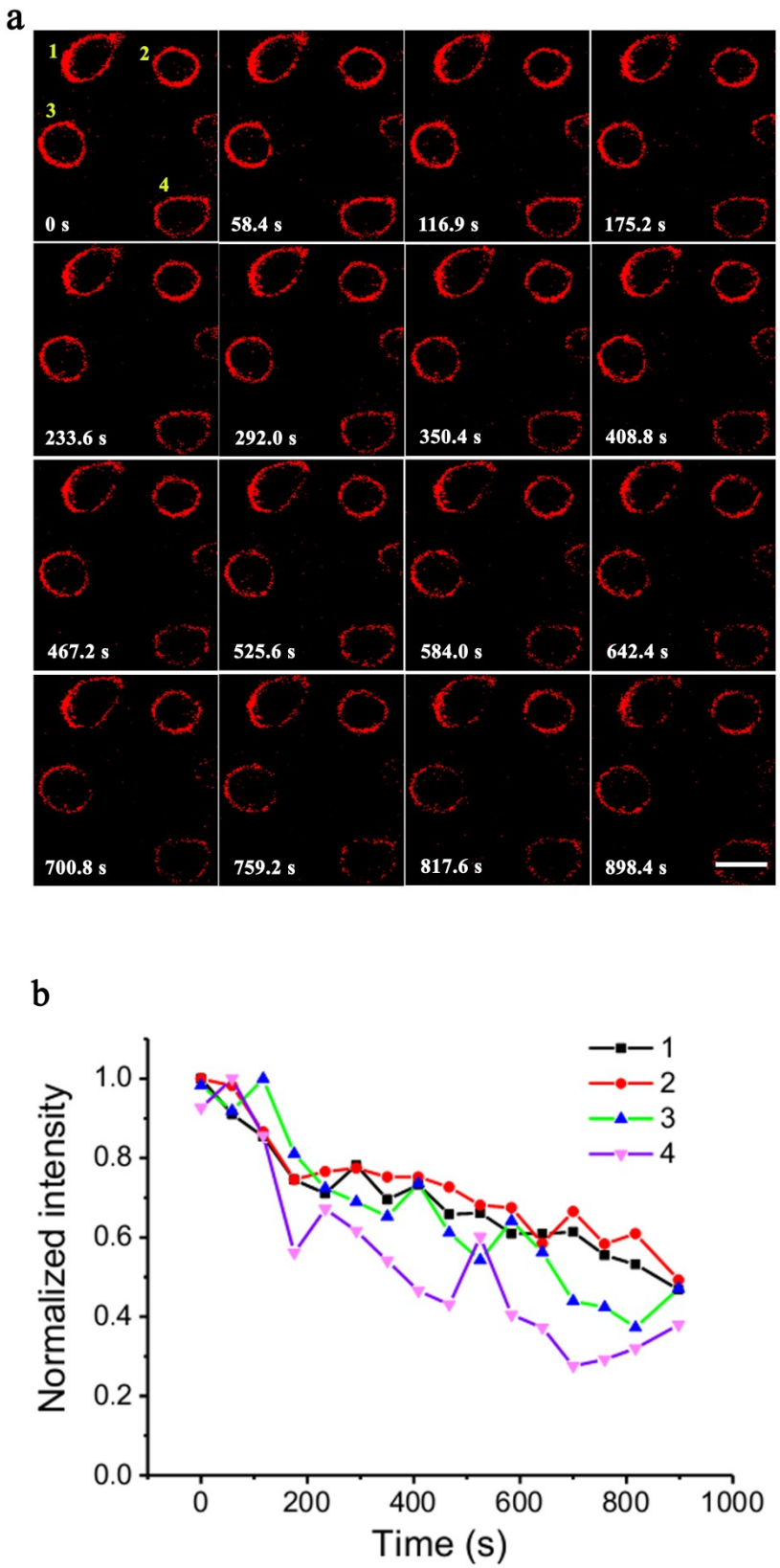

Fig S23. TPEF images of HeLa cells stained with **TSP** (a, 2 μM, 20 min) at different times under ceaseless exposure of laser source (3 mW) and the corresponding fluorescence intensity (b). λ_{ex} = 800 nm. Bar = 20 μm.

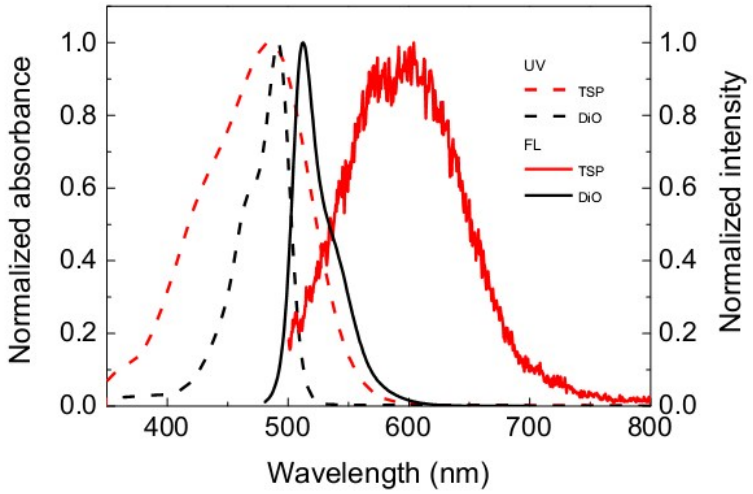

Fig S24. The absorption and fluorescence spectra of **TSP** (10 μM) in Gly and DiO (10 μM) in DMSO. λ_{ex} (**TSP**, DiO) = 488 nm.

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[4] X. Zhang, Y. M. Sun, X. Q. Yu, B. Q. Zhang, B. B. Huang and M. H. Jiang, *Synth. Met.*,2009, 159, 2491.

[5] N. S. Makarov, M. Drobizhev ,and A. Rebane, *Opt. Express*, 2008, 16, 4029.