Multicolor Monitoring of Cellular Organelles by Single Wavelength Excitation to Visualize Mitophagy Process

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Materials and methods

Materials and characterization: 9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABDA), 4-vinylpyridine, palladium(II) acetate, tri(*o*-tolyl)phosphine, trimethylamine, bromoethane, Cs_2CO_3, anhydrous dimethyl sulfoxide (DMSO), anhydrous N,N-dimethylformamide (DMF), boron tribromide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other chemicals were all purchased from Sigma-Aldrich and used as received without further purification. Tetrahydrofuran (THF) and dichloromethane were dried by distillation using sodium or calcium hydride as drying agent. All non-aqueous reactions were carried out under nitrogen atmosphere in oven-dried glassware. Deuterated solvents were purchased from Cambridge Isotope Laboratories Inc. Compound 1[1] and 3-morpholinopropyl 4-methylbenzenesulfonate[2] were synthesized according to reported literatures.

Dulbecco's Modified Essential Medium (DMEM) is a commercial product of Invitrogen. Milli-Q water was supplied by Milli-Q Plus System (Millipore Corporation, United States). Phosphate-buffer saline (PBS, 10×) buffer with pH = 7.4 is a commercial product of 1st BASE (Singapore). Milli-Q water (18.2 MΩ) was used to prepare the buffer solutions from the 10× PBS stock buffer. 1× PBS contains NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (10 mM), and KH_2PO_4 (1.8 mM). Hoechst 33342, FITC-tagged Annexin V, propidium iodide (PI), fetal bovine serum (FBS) were purchased from Life Technologies.

NMR spectra were measured on a Bruker ARX 400 NMR spectrometer. Chemical shifts are reported in parts per million referenced with respect to residual solvent ($CDCl_3$ = 7.26 ppm) for ^1H NMR and ($CDCl_3$ = 77.1 ppm) for ^{13}C NMR. The extent of reaction was monitored by thin layer chromatography (TLC) using Merck 60 F254 pre-coated silica gel plates with fluorescent indicator UV254. After the plates were subjected to elution in the TLC chamber. Flash column chromatography was carried out using Merck silica gel (0.040-0.063). Mass spectra were recorded on AmaZon X LC-MS for electrospray ionization (ESI). UV-vis absorption spectra were taken on a Shimadzu Model UV-1700 spectrometer. Photoluminescence (PL) spectra were measured on a Perkin-Elmer LS 55 spectrofluorometer. All UV and PL spectra were collected at 24 ± 1 °C. Zeta potentials were measured on a Zetasizer Nano ZS ZEN3600 analyzer (Malvern Instruments Ltd, UK) at 25 °C.

Synthesis of compound 2. Compound 1 (1.0 g, 2.2 mmol), 4-vinylpyridine (270 mg, 2.6 mmol), palladium(II) acetate (22 mg, 0.1 mmol), tri(*o*-tolyl)phosphine (61 mg, 0.2 mmol), trimethylamine (2 mL)

and DMF (8 mL) were mixed in a round-bottom flask, stirred and heated at 100 °C under argon atmosphere overnight. After cooling to room temperature, the reaction was quenched by water and ethyl acetate, then washed by water (20mL × 5). The organic phase was dried by Na_2SO_4 and the solvent was removed under reduced pressure. The obtained residue was separated with chromatography (hexane/ethyl acetate = 1/1) to give the desired product as greenish yellow solid (850 mg, 78%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.55 (d, J = 4.0 Hz, 2H), 7.32 (d, J = 5.9 Hz, 2H), 7.29 (s, 1H), 7.27 (s, 1H), 7.23-7.19 (m, 1H), 7.14-7.09 (m, 3H), 7.05-7.03 (m, 4H), 6.98-6.91 (m, 5H), 6.69 - 6.61 (m, 4H), 3.75 (s, 3H), 3.74 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.4, 158.3, 150.2, 145.3, 145.0, 144.2, 141.0, 138.7, 136.4, 136.3, 133.9, 133.2, 132.8, 132.7, 132.0, 131.6, 127.9, 126.6, 126.4, 125.5, 120.9, 113.3, 17, 55.2. ESI-MS, m/z: $[M+H]^+$ calcd 496.2, found 496.2.

Synthesis of AIE-Red. Compound 2 (90 mg, 0.18mmol) and bromoethane (110 mg, 1.1 mmol) were dissolved in DMF (5 mL) and heated at 80 °C under argon atmosphere for 14 h. After cooling to room temperature, the mixture was separated with chromatography directly (Eluent: hexane/ethyl acetate = 1/5, dichloromethane/methonal = 10/1) to get the desired product as orange solid (170 mg, 86 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.13 (d, J = 6.3 Hz, 2H), 8.00 (d, J = 6.5 Hz, 2H), 7.64 (d, J = 16.2 Hz, 1H), 7.35 (d, J = 8.1 Hz, 2H), 7.11-6.97 (m, 8H), 6.95-6.88 (m, 4H), 6.66-6.58 (m, 4H), 4.80 (dd, J = 14.2, 6.9 Hz, 2H), 3.72-3.70 (m, 6H), 1.64 (t, J = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.4, 158.3,, 150.2, 145.3, 145.0, 144.2, 141.0, 138.7, 136.4, 136.3, 133.9, 133.2, 132.8, 132.7, 132.0, 131.6, 127.9, 126.6, 126.4, 125.5, 120.9, 113.3, 113.2, 56.4, 55.2, 17.1. ESI-MS, m/z: $[M-Br]^+$ calcd 524.2590, found 524.2588.

Synthesis of AIE-Green.

(i) (*E*)-4,4'-(2-phenyl-2-(4-(2-(pyridin-4-yl)vinyl)phenyl)ethene-1,1-diyl)diphenol: Compound 2 (270 mg, 0.54 mmol) was dissolved in anhydrous dichloromethane and stirred at 0 °C under argon atmosphere. Boron tribromide (0.51 mL, 5.4 mmol) was added dropwise into the solution in 10 min. After recovering to room temperature, the mixture was stirred overnight. The reaction was quenched by water and extracted by dichloromethane, then washed by water (20mL × 3). The organic phase was dried by Na_2SO_4 and the solvent was removed under reduced pressure to get the desired product (243 mg, 0.52 mmol), which was directly used without further purification.

(ii) **AIE-Green**: (*E*)-4,4'-(2-phenyl-2-(4-(2-(pyridin-4-yl)vinyl)phenyl)ethene-1,1-diyl)diphenol (100 mg, 0.21 mmol) ,3-morpholinopropyl 4-methylbenzenesulfonate (150 mg, 0.5 mmol), Cs_2CO_3 (195 mg, 0.6 mmol) and DMF (5 mL) were mixed and heated at 60 °C for 8 h. After cooling to room temperature, the reaction was quenched by water and ethyl acetate, then washed by water (20mL × 5). The organic phase was dried by Na_2SO_4 and the solvent was removed under reduced pressure. The obtained residue was

separated with chromatography (dichloromethane/methanol = 100/1) to give the desired product as greenish yellow solid (118 mg, 78%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.54 (d, J = 5.8 Hz, 2H), 7.31-7.27 (m, 4H), 7.20 (d, J = 16.3 Hz, 1H), 7.12-7.09 (m, 3H), 7.03 (d, J = 8.0 Hz, 4H), 6.96-6.89 (m, 5H), 6.63 (t, J = 9.1 Hz, 4H), 3.98-3.92 (m, 4H), 3.75-3.72 (m, 8H), 2.56-2.47 (m, 12H), 1.99-1.92 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 157.7, 150.2, 145.5, 144.9, 144.1, 140.9, 138.7, 133.8, 133.1, 132.7, 132.0, 131.5, 127.9, 126.5, 125.5, 120.9, 113.8, 113.7, 66.8, 65.9, 55.7, 53.7, 26.3. ESI-MS, m/z: $[M+H]^+$ calcd 722.3880, found 722.3964.

Cell Culture. Human cervix carcinoma HeLa cells were provided by American Type Culture Collection (ATCC). The cells were cultured in DMEM medium containing 100 μg mL^{-1} streptomycin, 10% heat-inactivated FBS, 100 U mL-1 penicillin, and maintained in a humidified incubator with 5% CO_2 at 37 °C.

Confocal imaging. The cells were cultured in 8-well chambers and precultured overnight. Then the medium was replaced with fresh one and incubated with the **AIE-Red** (5 μM), **AIE-Green** (5 μM), Mito Tracker (150 nM), Lyso Tracker (100 nM), fluorescein diacetate (FDA) and propidium iodide (PI). The cells were imaged by confocal laser scanning microscope (CLSM, Zeiss LSM 410, Jena, Germany). The images were analyzed by Image J 1.43 × program (http://rsbweb.nih.gov/ij/). (**AIE-Red**: E_x = 405 nm, E_m = 650-700 nm; Mito Tracker: E_x = 488 nm, E_m = 505-525 nm; **AIE-Green**: E_x = 405 nm, E_m = 505-525 nm; Lyso Tracker: E_x = 570 nm, E_m = 650-700 nm; Hoechst 33342: E_x = 405 nm, E_m = 430-470 nm; FDA: E_x = 488 nm, E_m = 510-530 nm; PI: E_x = 570 nm, E_m = 650-700 nm).

Cytotoxicity studies. The metabolic activity of the cells was assessed by 3-(4,5- dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. After incubation of the cells in DMEM medium overnight, the medium was removed, washed with PBS and the cells were incubated with TPE-Red, TPE-Green, Mito Tracker or Lyso Tracker at different concentrations for 24 h. The cells were further washed with 1× PBS before the addition of 100 μL of MTT solution (0.5 mg mL^{-1}) into each well. After 3 h incubation, the MTT solution was removed and DMSO (100 μL) was added into each well. The absorbance of MTT at 570 nm was studied by microplate reader (Genios Tecan). The cells without any treatment were used as control.

Zebrafish Line. Four to five pairs of zebrafish were placed in crossing tanks for spawning overnight. Embryos were settled to the bottom of the tank, and were collected using a sieve and transferred to petri dishes for embryo culture. They were screened, incubated at 27 ^{o}C, 0.4% CO_2 and grown in egg water (10 % NaCl; 1.63 % $MgSO_4 \cdot 7H_2O$; 0.4 % $CaCl_2$; 0.3 % KCl). After 22 h postfertilization, PTU was added to prevent melanin formation to yield optically transparent fish. 3 d postfertilization the embryos were seeded to into 96 well plates at 1 embryo per well. The embryos were soaked in AIE-Red, AIE-Green, Mito Tracher or Lyso Tracker for 30 min and were imaged for uptake using confocal imaging (Carl Zeiss LSM 510 Meta)

using 405 nm, 488 nm or 570 nm. (**AIE-Red**: E_x = 405 nm, E_m = 650-700 nm; **AIE-Green**: E_x = 405 nm, E_m = 505-525 nm).

1. D. Jana and B. K. Ghorai, *Tetrahedron Letters*, 2012, **53**, 6838-6842.
2. J. M. Shin, G. Sachs, Y.-m. Cho and M. Garst, *Molecules*, 2009, **14**, 5247-5280.

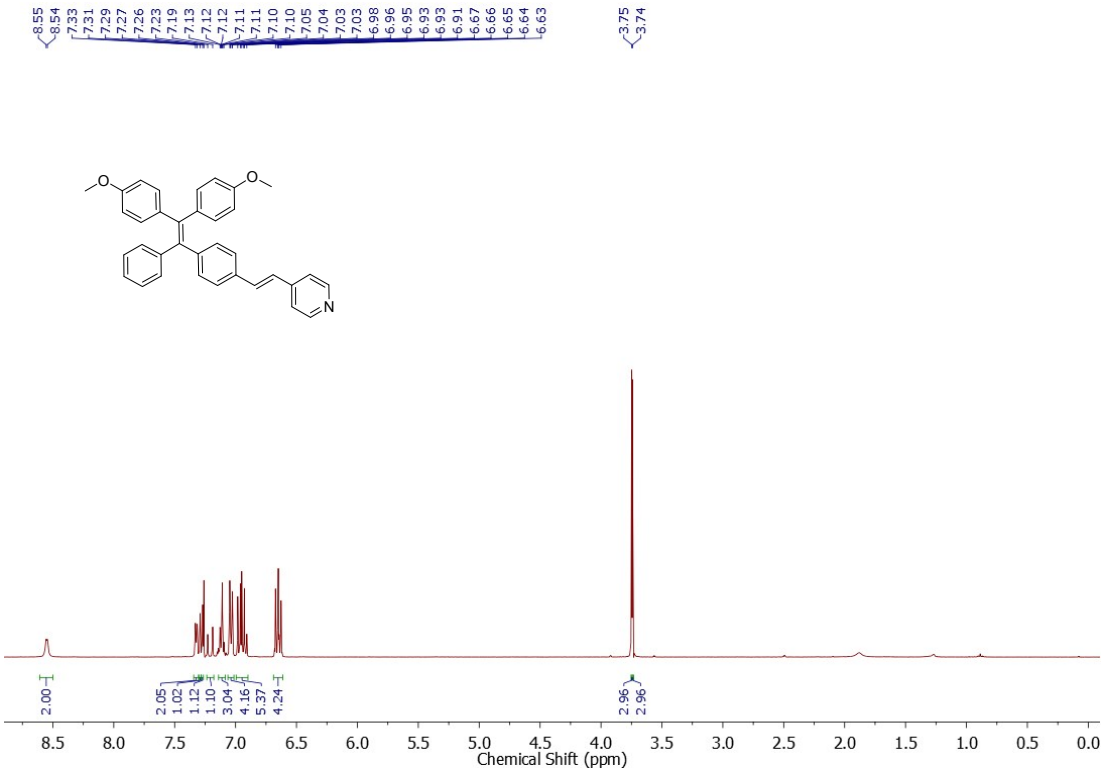

Figure S1. ^{1}H NMR spectrum of compound **2** in $CDCl_3$.

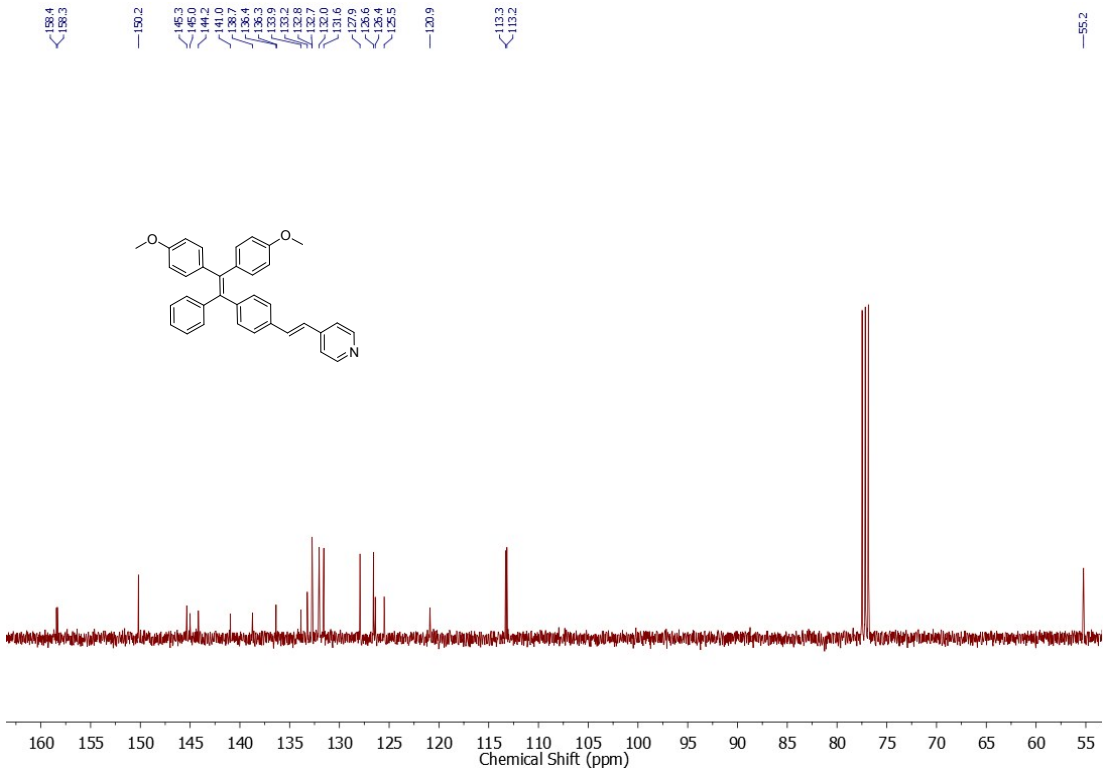

Figure S2. ^{13}C NMR spectrum of compound **2** in $CDCl_3$.

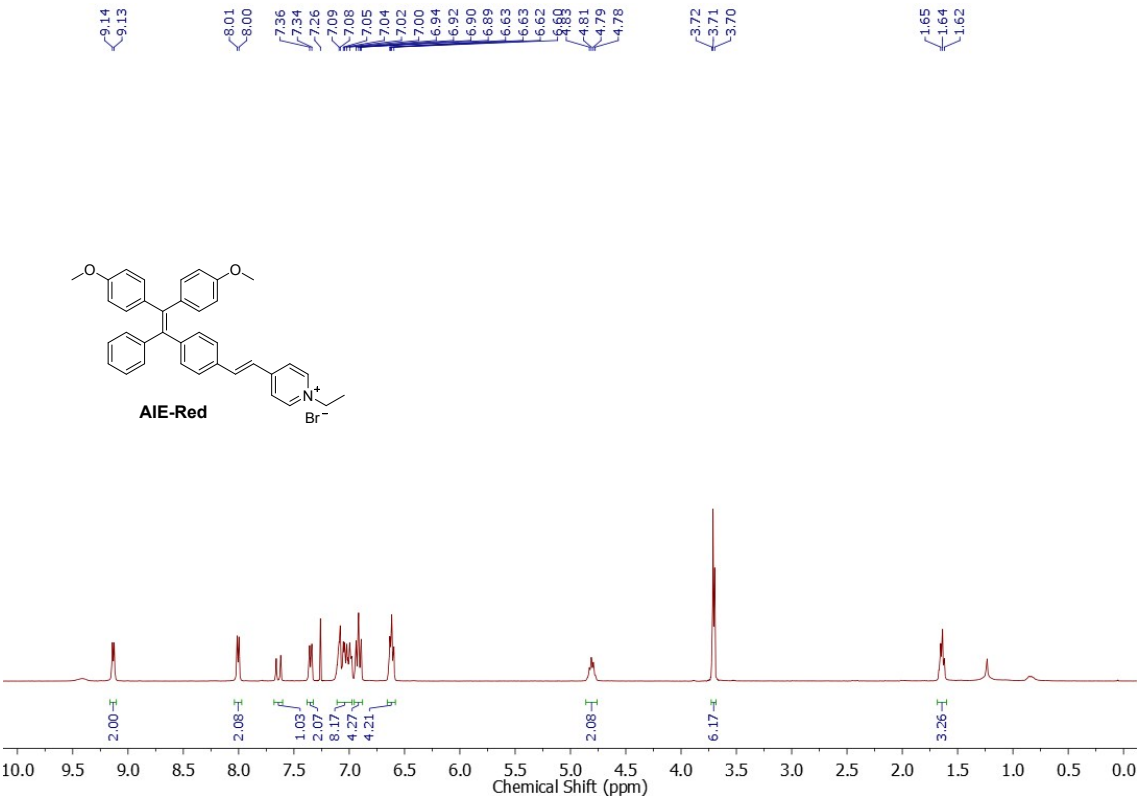

Figure S3. ^{1}H NMR spectrum of **AIE-Red** in $CDCl_3$.

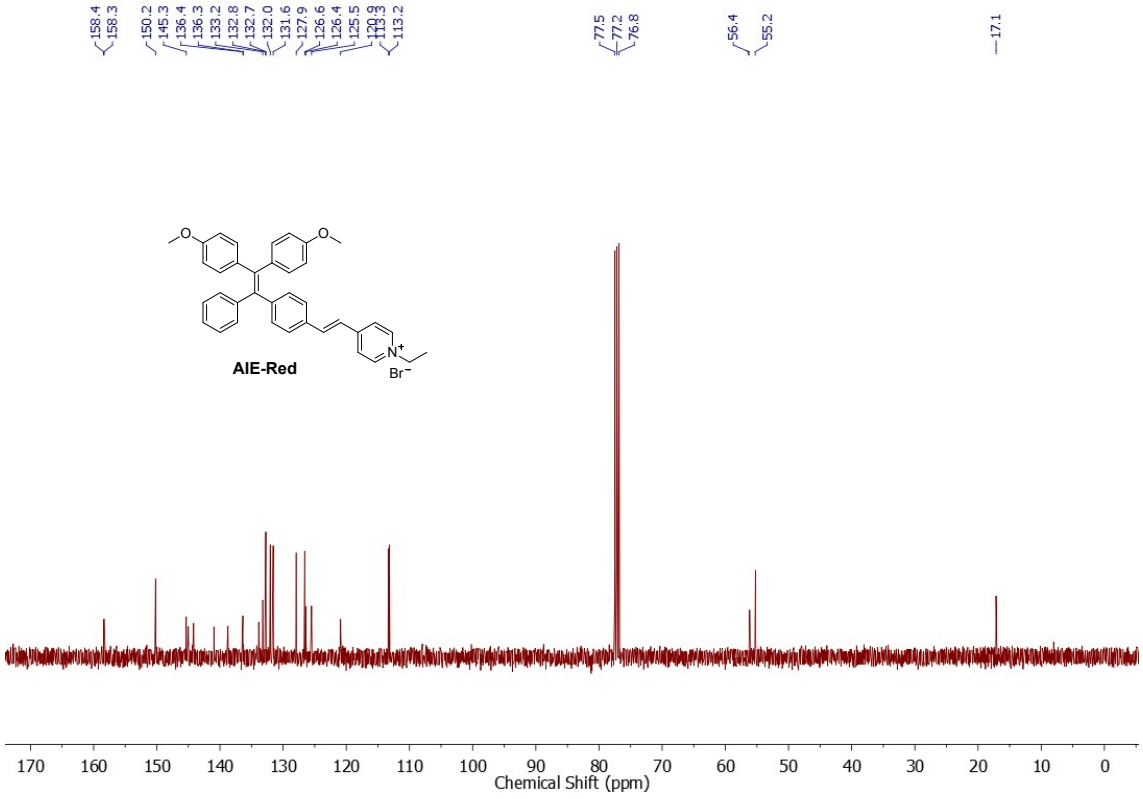

Figure S4. ^{13}C NMR spectrum of **AIE-Red** in $CDCl_3$.

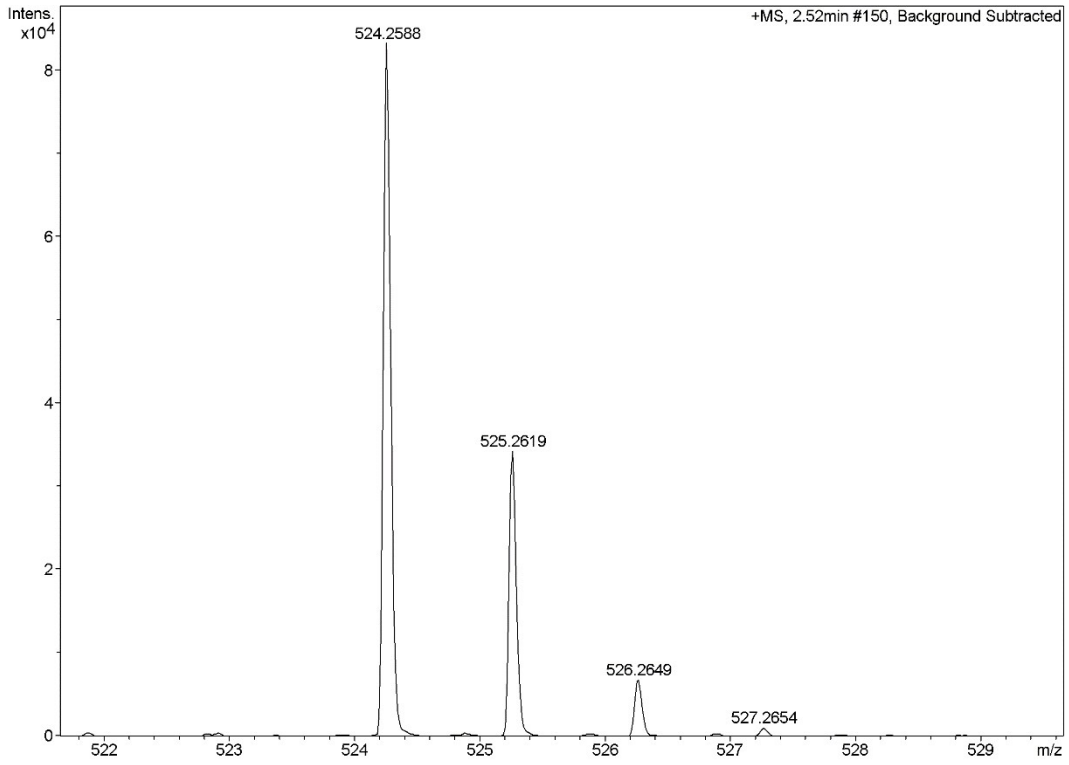

Figure S5. High resolution mass spectrum of **AIE-Red**.

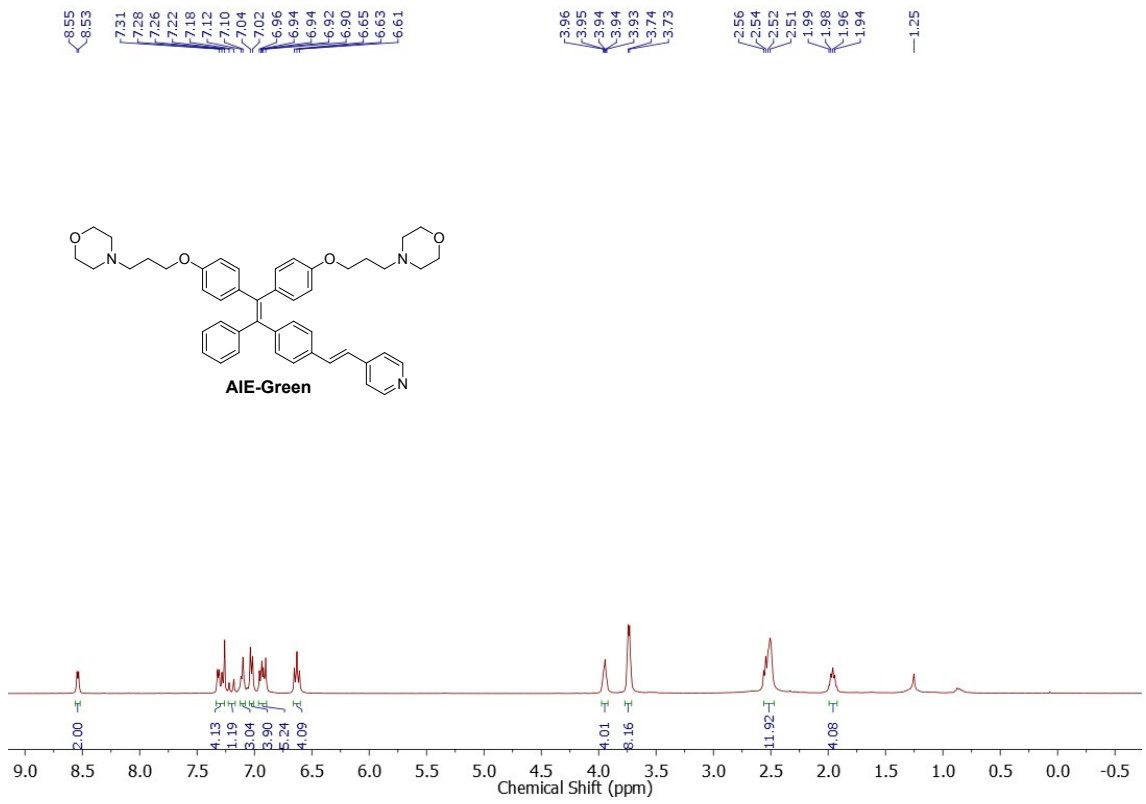

Figure S6. ^{1}H NMR of **AIE-Green** in $CDCl_3$.

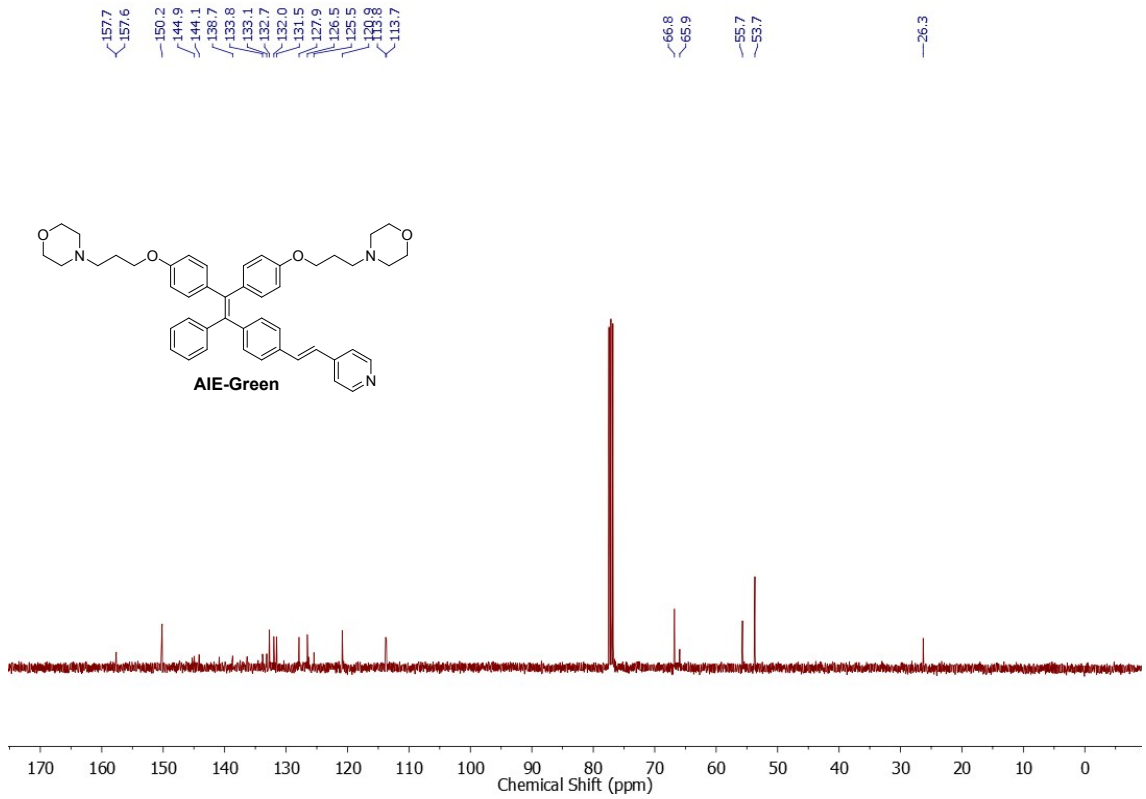

Figure S7. ^{13}C NMR of **AIE-Green** in $CDCl_3$.

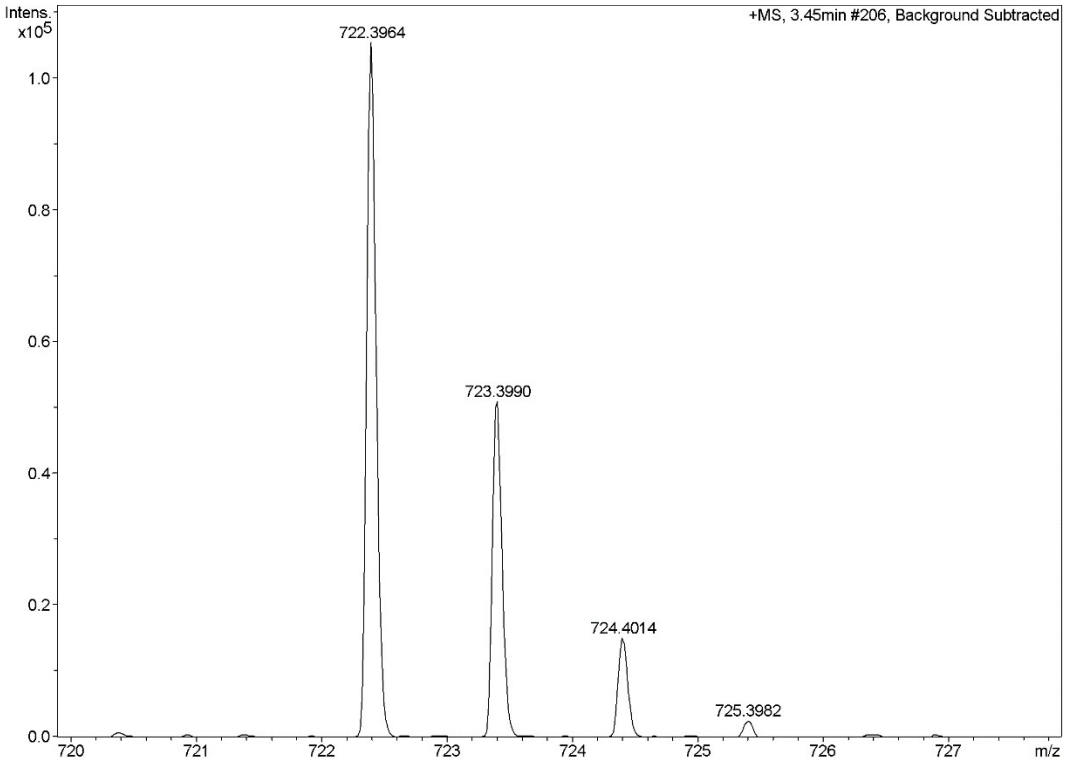

Figure S8. High resolution mass spectrum of **AIE-Green**.

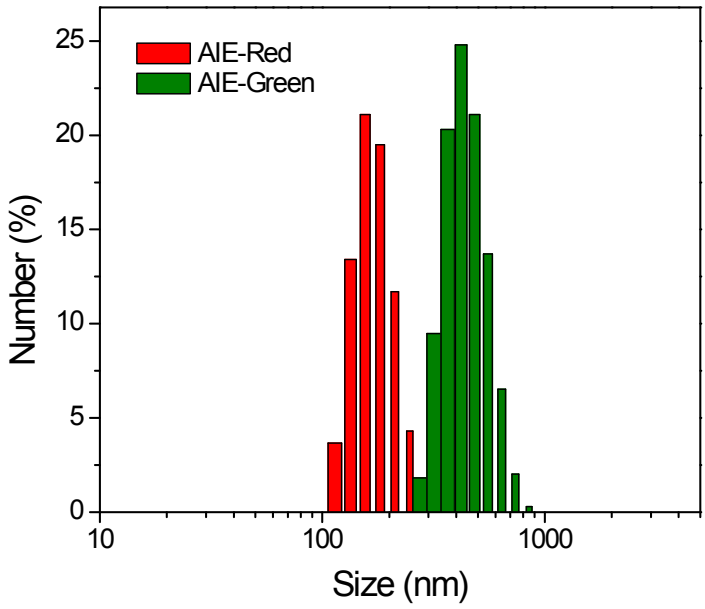

Figure S9. The size distributions of **AIE-Red** (5 μM) and **AIE-Green** (5 μM) in solution of PBS/DMSO (99/1, v/v).

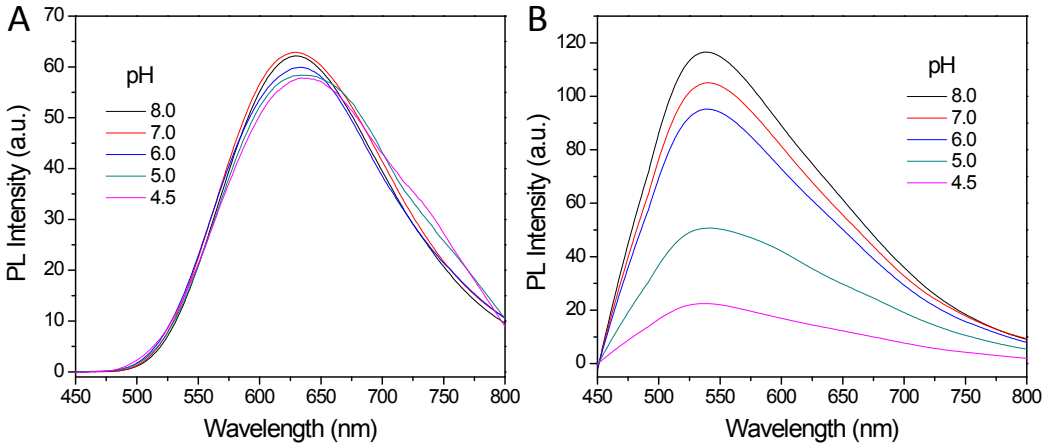

Figure S10. The fluorescence spectra of AIE-Red (10 μM) (A) and AIE-Green (10 μM) (B) in PBS with different pH ranging from 8.0 to 4.5.

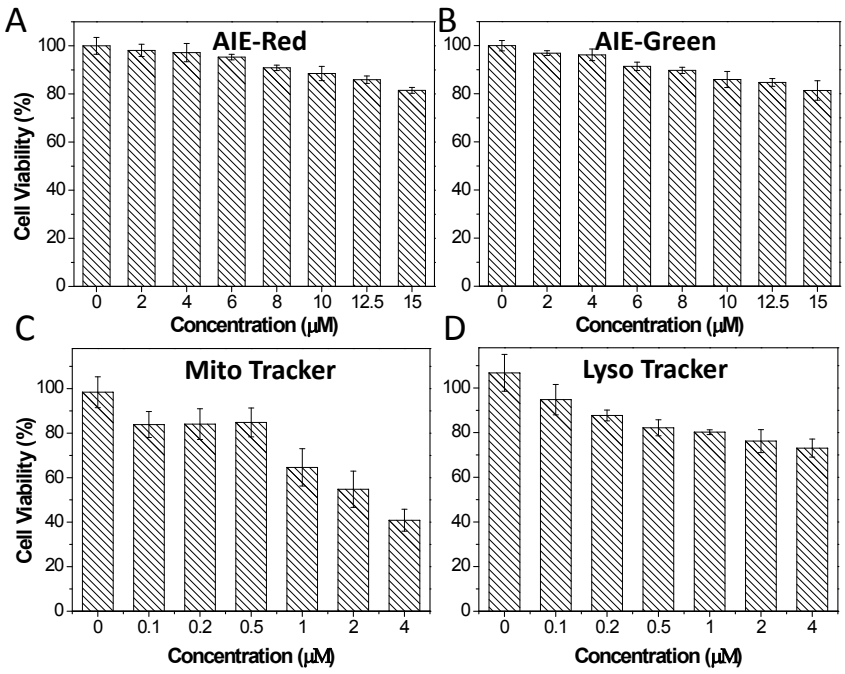

Figure S11. HeLa cell viability after treatment by **AIE-Red** (A), **AIE-Green** (B), Mito Tracker (C) and Lyso Tracker (D) at different concentrations.

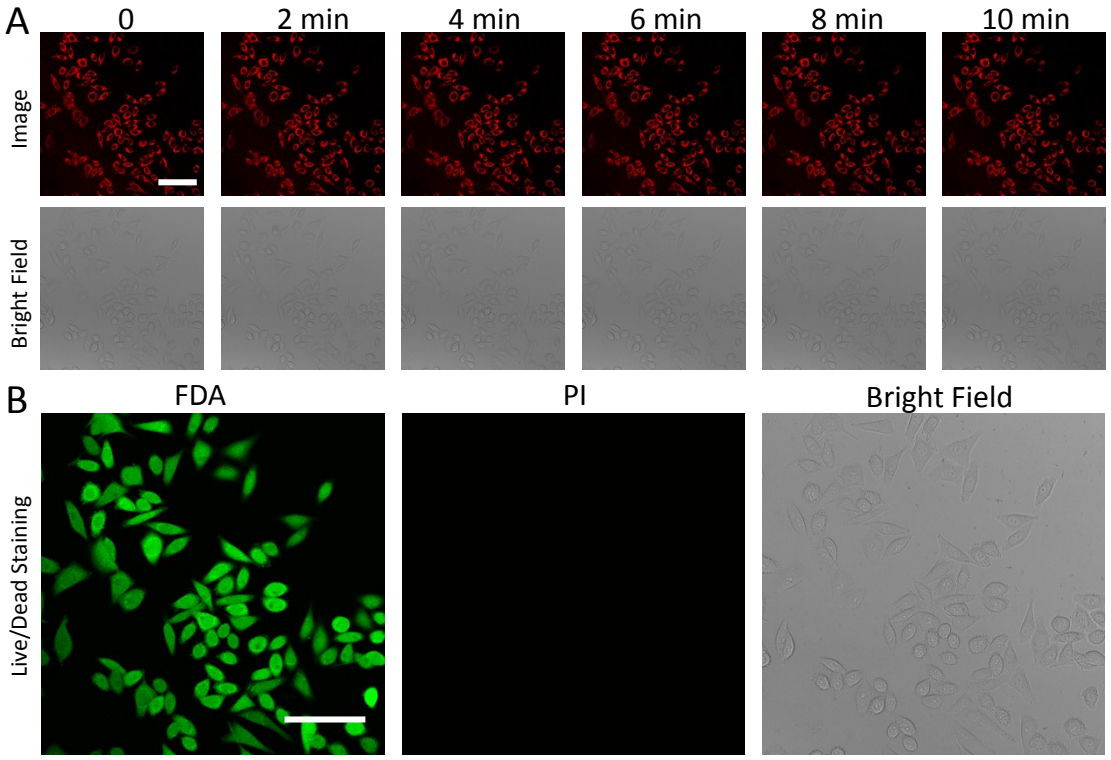

Figure S12. (A) Confocal images and the bright field images of **AIE-Red** (5 μM) incubated HeLa cells upon continuous confocal laser irradiation for 10 min. (B) Live/Dead assay of **AIE-Red** (5 μM) incubated HeLa cells after 10 min continuous confocal laser irradiation. The scale bar is 100 μm.

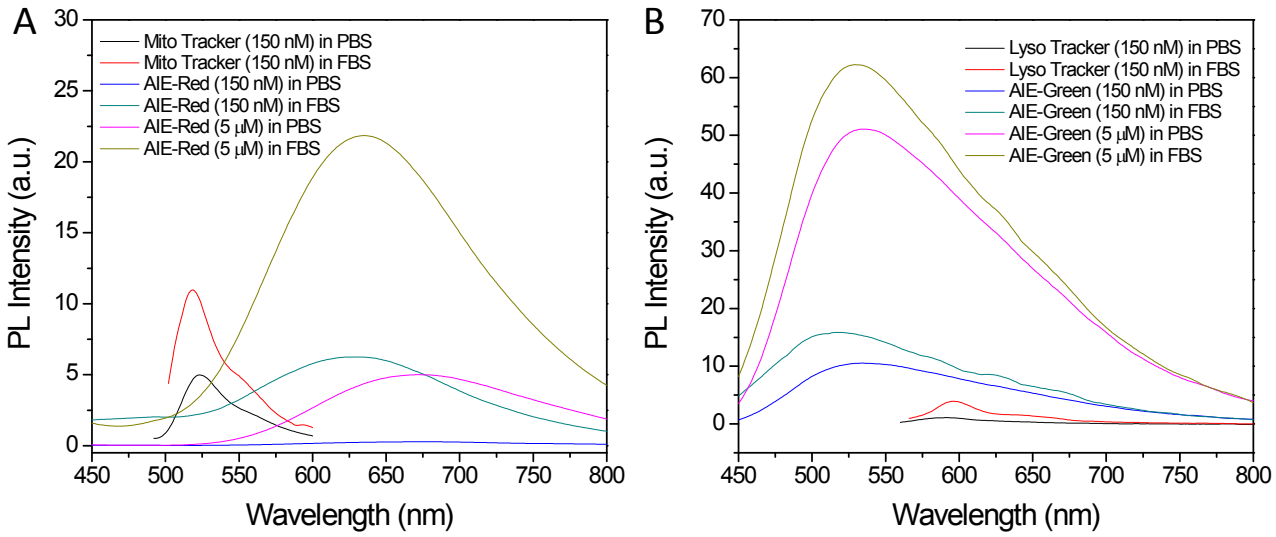

Figure S13. The fluorescence spectra of **AIE-Red**, Mito Tracker (A) and **AIE-Green**, Lyso Tacker at different concentrations in PBS or FBS-containing PBS. (The excitation wavelengths of **AIE-Red**, Mito Tracker, **AIE-Green** and Lyso Tracker are 405 nm, 488 nm, 405 nm, 570 nm, respectively)

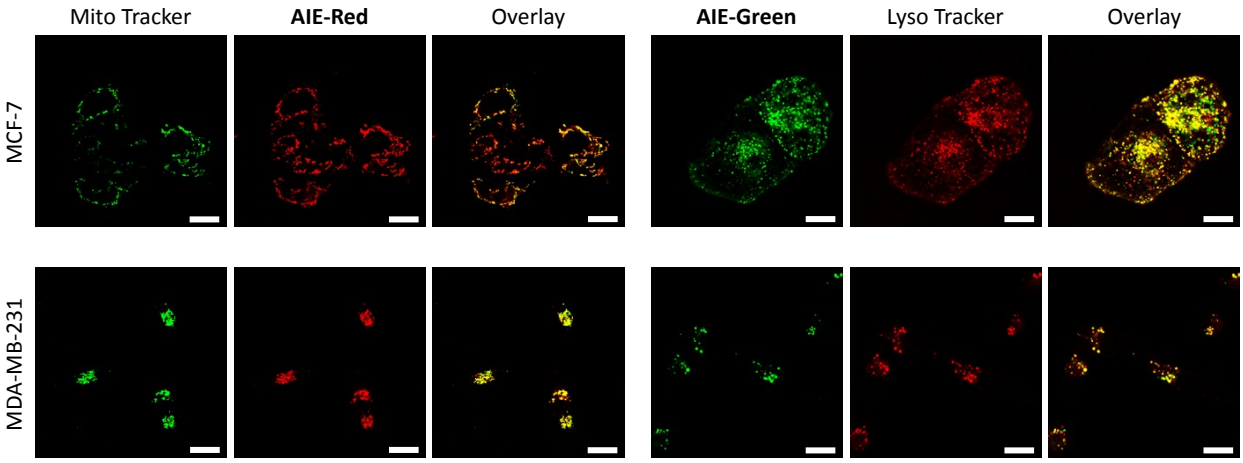

Figure S14. **.** Confocal images of MCF-7 and MDA-MB-231 cells after incubation with **AIE-Red** (5 μM), Mito Tracker (150 nM) and the overlay image; **AIE-Green** (5 μM), Lyso Tracker (100 nM) and the overlay image. The scale bar is 30 μm.