Supporting Information

An Intracellular Anchor Regulates Distribution of Bioactive Molecules

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Materials

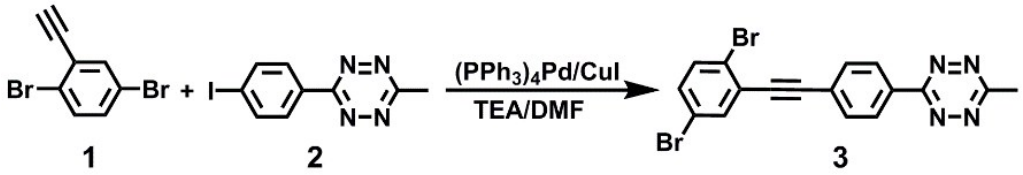
All the commercial available chemical reagents were purchased from Acros, Aldrich Chemical Company or Alfa-Aesar and used without further purification. All organic solvents were supplied by Beijing Chemical Works and used as received. Water was purified by a Millipore filtration system. TCO-$(PEG)_4$-NHS was obtained from Click Chemistry Tools. Compounds **1** and **2** were synthesized according to the procedures of literatures.[1,2] MCF-7 cells, HeLa cells and A549 cells were obtained from cell culture center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). Fetal bovine serum (FBS) was purchased from Sijiqing Biological Engineering Materials (Hangzhou, China). Dulbecco's modified Eagle medium (DMEM), phosphate buffer saline (PBS) and Hanks' balanced salt solution with Ca^{2+} & Mg^{2+} (HBSS with Ca^{2+} & Mg^{2+}) were purchased from Hyclone (Beijing, China). Lyso-Tracker® Deep Red, Mito-Tracker® Deep Red FM and ER-Tracker™ Red were purchased from Life Technologies (Beijing, China). Golgi-Tracker Red was purchased from Beyotime Institute of Biotechnology (Jiangsu, China)

Instruments:

The ^{1}H NMR and ^{13}C NMR spectra were obtained from Bruker Avance 300 MHz, 400 MHz or 500 MHz spectrometer. Mass spectra were recorded on a Bruker 9.4T Solarix FT-ICR-MS for high resolution mass spectra (HR-MS). UV-Vis absorption spectra were recorded on a JASCOV-550 spectrophotometer. Fluorescence spectra were measured on a Hitachi F-4500 fluorimeter equipped with xenon lamp excitation source. MTT analysis was recorded on a microplate reader (BIO-TEK Synergy HT, USA) at a wavelength of 570 nm. Cell counting was conducted at an automated cell counter (Countess, Invitrogen). Confocal laser scanning microscopy (CLSM) images were obtained from confocal laser scanning biological microscope (FV1000-IX81, Olympus, Japan). High performance liquid chromatography (HPLC) was performed on a Waters 2535Q system, using SunFire™ C18 (5 μm, 10 mm × 250 mm) semi-preparative column and SunFire™ C18 (5 μm, 10 mm × 250 mm) analytical column.

Synthesis

Synthesis of 3-(4-((2,5-dibromophenyl)ethynyl)phenyl)-6-methyl-1,2,4,5-tetrazine (compound 3)

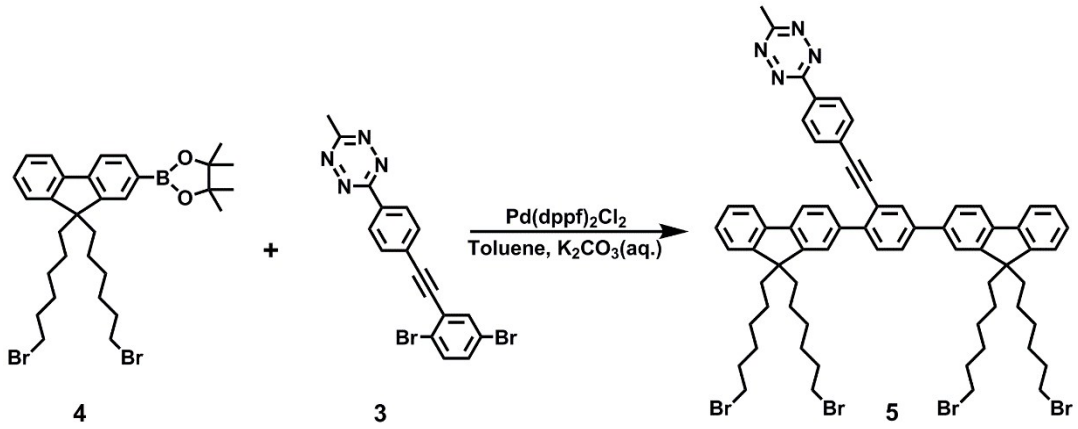


1 2 3

Compound **1** (618 mg, 2.4 mmol), compound **2** (628 mg, 2.1 mmol), Tetrakis-(triphenylphosphine) palladium (138 mg 0.12 mmol), copper(I) iodide (40 mg, 0.21 mmol), triethylamine (3 mL) and *N*, *N*-dimethyl formamide were added to a 100 mL two-neck flask and degassed with argon for 30 minutes. The mixture were stirred at 60°C for 2 h, then at room temperature overnight under argon atmosphere and the solvent was evaporated under vacuum. The residue was dissolved in chloroform, washed with

distilled water and dried over anhydrous $MgSO_4$. The solvent was removed in vacuo and the residue was subjected to silica gel chromatography with petroleum/ethyl acetate (25:1) as eluent to afford magenta solid (654 mg, 73%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.67 - 8.57 (m, 2H), 7.83 - 7.70 (m, 3H), 7.50 (d, J = 8.5 Hz, 1H), 7.34 (dd, J = 8.6, 2.4 Hz, 1H), 3.12 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ167.44, 163.77, 135.92, 133.92, 133.03, 132.66, 132.09, 127.95, 126.92, 124.59, 120.86, 94.48, 89.77, 21.35. HRMS (MALDI): calcd for $[C_{17}H_{11}Br_2N_4]^+$ ($[M+H]^+$) 428.93450, found 428.93481.

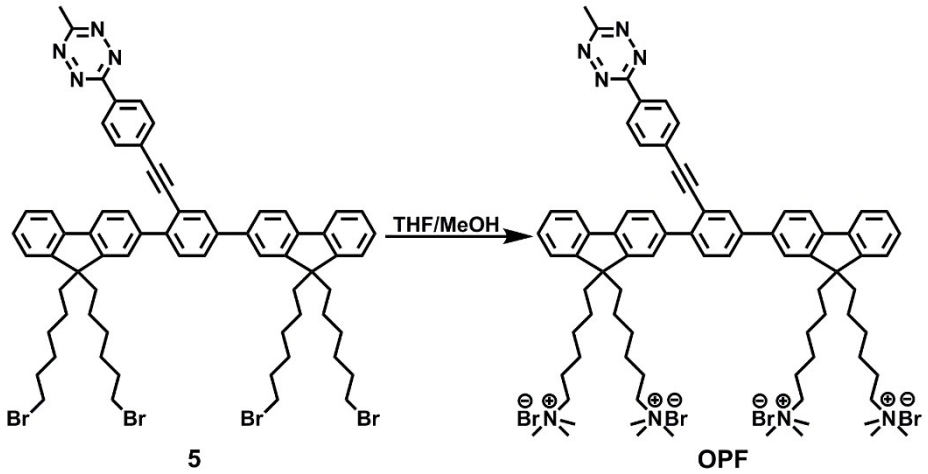
Synthesis of 3-(4-((2,5-bis(9,9-bis(6-bromohexyl)-9H-fluoren-2-yl)phenyl)ethynyl)phenyl)-6-methyl-1,2,4,5-tetrazine (5)



To a solution of compound **3** (64 mg, 0.15 mmol) and compound **4** (195 mg, 0.32 mmol) in toluene (14 mL) was added aqueous sodium carbonate (2 mol/L, 7 mL). The mixture was degassed with argon for 30 minutes, added with 1,1'–Bis(diphenylphosphino)-ferrocene-palladium(II) dichloride dichloromethane complex (5 mg) under argon atmosphere and stirred at 90°C for 24 hours. After cooled to room temperature, 100 mL of chloroform was added. The organic solution was filtrated, washed with water (50 mL×3), dried over anhydrous $MgSO_4$ and concentrated under vacuum. The obtained organic residue was purified by silica gel column chromatography with petroleum/ethyl acetate (20:1) as eluent to afford pink solid (76 mg, 40%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.58 - 8.49 (m, 2H), 8.03 (d, J = 2.4 Hz, 1H), 7.89 - 7.51 (m, 12H), 7.42 - 7.31 (m, 6H), 3.23 (dt, J = 31.4, 6.8 Hz, 8H), 3.09 (s, 3H), 2.09 -

1.96 (m, 8H), 1.75 - 1.46 (m, 8H),1.28 - 0.84 (m, 16H), 0.80 - 0.60 (m, 8H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.33, 163.76, 151.36, 150.82, 150.74, 150.53, 143.37, 141.07, 140.88, 140.77, 140.59, 139.20, 138.80, 132.35, 131.96, 131.38, 130.49, 128.33, 128.01, 127.87, 127.47, 127.39, 127.16, 126.16, 123.82, 123.07, 122.98, 121.62, 121.36, 120.32, 120.06, 119.46, 93.02, 91.68, 77.36, 55.28, 55.16, 40.41, 40.25, 34.07, 33.98, 32.78, 32.76, 29.84, 29.19, 27.93, 27.90, 23.84, 23.72, 21.32. HRMS (MALDI): calculated for $[C_{67}H_{74}Br_4N_4]^+$ ($[M]^+$) 1248.24850, found 1248.24823.

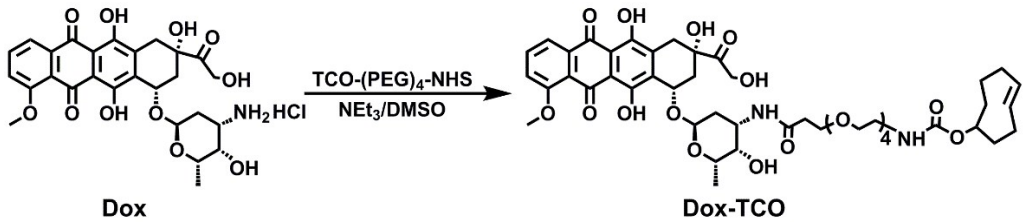
Synthesis of OPF



Compound **5** (33 mg, 0.026 mmol) was dissolved in THF (8 mL) and added with trimethylamine in methanol solution (3.2 mol/L, 6 mL) under the ice-water bath. The mixture was stirred at 40°C for 24 hours. After the reaction, the solvent and excess trimethylamine were removed by vacuum distillation to obtain pink solid (35.6 mg, 92%). ^{1}H NMR (400 MHz, MeOD) δ 8.51 (d, J = 8.3 Hz, 2H), 8.07 (s, 1H), 7.97 - 7.86 (m, 3H), 7.86 - 7.73 (m, 5H), 7.72 - 7.65 (m, 4H), 7.53 - 7.28 (m, 6H), 3.26 - 3.10 (m, 8H), 3.05 (s, 3H), 3.03(d, J = 2.8 Hz, 36H), 2.32 - 1.96 (m, 8H), 1.62 - 1.44 (m, 8H), 1.28 - 1.09 (m, 8H), 1.08 - 0.94 (m, 8H), 0.80 - 0.52 (m, 8H). ^{13}C NMR (125 MHz, MeOD) δ 168.95, 165.00, 152.58, 151.88, 151.42, 144.37, 142.68, 142.31, 142.19, 141.57, 140.48, 139.77, 133.39, 132.55, 131.66, 129.42, 129.00, 128.71, 128.52, 128.29, 127.11, 124.71, 124.05, 122.61, 122.19, 121.40, 121.03, 120.69, 93.54,

92.47, 67.66, 56.49, 56.36, 53.45, 41.20, 35.37, 30.36, 30.24, 27.02, 26.87, 24.93, 24.75, 23.71, 23.59, 21.17. HRMS (ESI): calculated for $[C_{79}H_{108}N_8]^{4+}$ ($[M-4Br]^{4+}$) 292.21688, found 292.21696.

1.2.2 Synthesis of Dox-TCO



The Doxorubicin hydrochloride (16 mg, 0.028 mmol) and TCO-$(PEG)_4$-NHS (14 mg, 0.028 mmol) was dispersed in anhydrous DMSO (5 mL), followed by adding triethylamine (15 μL). The mixture was stirred in the dark at room temperature for 24 hours. After the reaction, 100 mL of dichloromethane was added and the obtained solution was washed with water (50 mL×5) to remove DMSO. The organic phase was dried over anhydrous Na_2SO_4 and evaporated under vacuum to obtain the crude product, which was purified by semi-preparative RP-HPLC with methanol and water as the eluent to yield pink solid (24.8 mg, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.95 (s, 1H), 13.23 (s, 1H), 8.02 (d, J = 7.7 Hz, 1H), 7.77 (t, J = 8.1 Hz, 1H), 7.37 (d, J = 8.5 Hz, 1H), 6.72 (s, 1H), 5.80 - 5.44 (m, 3H), 5.44 - 5.22 (m, 2H), 4.77 (s, 2H), 4.39 - 4.28 (m, 1H), 4.26 - 3.97 (m, 4H), 3.83 - 3.41 (m, 17H), 3.41 - 3.23 (m, 3H), 3.13 - 2.83 (m, 2H), 2.54 - 2.30 (m, 4H), 2.30 - 1.40 (m, 13H), 1.39 - 1.14 (m, 4H), 0.98 - 0.79 (m, 1 H) ^{13}C NMR (75 MHz, $CDCl_3$) δ 214.18, 187.18, 186.77, 171.10, 161.15, 156.51, 156.42, 155.83, 135.84, 135.65, 135.04, 133.81, 133.12, 121.04, 121.04, 119.97, 118.54, 111.65, 111.47, 101.17, 80.65, 77.36, 70.68, 70.60, 70.27, 69.71, 68.90, 67.59, 65.73, 56.80, 45.34, 41.34, 40.96, 40.93, 38.84, 37.33, 35.81, 34.42, 34.11, 32.67, 31.11, 29.83, 17.17. HRMS (MALDI): calculated for $[C_{47}H_{62}N_2NaO_{18}]^+$ ($[M+Na]^+$) 965.38898, found 965.38902.

Reaction in aqueous and verification through HPLC and HR-MS

OPF and Dox-TCO were dissolved in aqueous (with less than 1% DMSO to improve the solubility) to a final concentration of 200 μmol/L and 400 μmol/L respectively. The aqueous solution of Dox-TCO was added to the OPF aqueous solution and stirred at 37 °C for 4 hours. After the reaction, the solvent was removed by vacuum freeze drying. The residue was subjected to C18 semi-preparative HPLC to preliminarily purification to obtain OPF-Dox. The two reactants OPF and Dox-TCO, and the product OPF-Dox were dissolved in methanol and subjected to C18 analytical HPLC and record the retention for each specimen. The time/gradient of the elution system is conducted as follows: solvent A is water, and solvent B is methanol, percentage of solvent B: 0 min, 0%; 5 min, 0%; 45 min, 100%; 70 min, 100%. The absorbance of the elute stream was detected at the wavelength of 325 nm for OPF and OPF-Dox, and 496 nm for Dox-TCO. The liquid collected over the duration of the peak for each sample was subjected to mass spectra characterization to further confirm the reaction.

Characteristic of optical properties

Investigation of the UV-*vis* absorption and fluorescence emission properties of Dox-TCO, OPF and OPF-Dox: First, the absorption and emission spectra of aqueous solution of Dox-TCO, OPF and OPF-Dox were recorded. The concentration of Dox-TCO was 30 μmol/L, and both OPF and OPF-Dox was measured at the concentration of 20 μmol/L.

Cell culture

MCF-7 cells, HeLa cells and A549 cells were cultured in Dulbecco's modified Eagles medium (DMEM) with 10% fetal bovine serum (FBS) at 37 °C under 5% CO_2 atmosphere.

Cell viability assay

MCF-7 cells, HeLa cells and A549 cells were seeded in 96-well plates at a density of 5000 cells/well and cultured for 12 hours. Then, the cells were incubated with various concentration of compounds (OPF, Dox-TCO) in fresh medium, controlled groups in the absence of aforementioned compounds was set as well. After incubation for specific time (24 hours for OPF and 4 h for Dox-TCO), the culture medium was replaced with DMEM containing methylthiazolyldiphenyl-tetrazolium bromide (MTT, 0.5 mg/mL) and cultured for another 4 hours. The medium was abandoned and DMSO (100 μL per well) was added to dissolve the produced formazan. The plate was shaken for 3 min and the absorption at 570 nm was recorded. The cell viability (VR) was determined by the following equation:

$$VR(\%) = \frac{A}{A_0} \times 100\%$$

Where A is the absorbance values of group added with OPF or Dox-TCO, and A_0 is the average absorbance value of the control group.

Intracellular location analysis of OPF

MCF-7 cells were seeded in confocal dishes at a density of 1.5×105 per plate and cultured for 12 h. After the adherence, cells were incubated with medium containing OPF (10 μmol/L) for 24 h after removing OPF in medium, Lyso-Tracker® Deep Red (0.5 μmol/L) and Mito-Tracker® Deep Red FM (0.5 μmol/L) in culture medium were added respectively and incubated for another 10 min to label lysosomes and mitochondria. ER-Tracker™ Red (1.0 μmol/L) in culture medium was applied to staining endoplasmic reticulum for 30 min. Golgi-Tracker Red was conducted following protocol procedures to stain Golgi apparatus. Nucleus staining is conducted as following procedures: MCF-7 cells were fixed by paraformaldehyde (4% in PBS) at 37 °C for 20 min, subsequently incubated with

RNA polymerase (5 mg/mL in PBS) for 30 min to remove RNA in cytoplasm, and lastly stained with PI (50 μg/mL in PBS) at 37 °C for 20 min. All specimens treated with organelle Trackers were washed with PBS twice and examined by CLSM. The co-localization of OPF with Lyso-Tracker in A549 and HeLa cells was also conducted on the same conditions. The excited wavelengths were 405 nm for OPF, 559 nm for ER-Tracker, Golgi-Tracker and PI, and 635 nm for Lyso-Tracker and Mito-Tracker.

Regulating the distribution of Dox-TCO through intracellular reaction with OPF

The adherent MCF-7 cells in confocal dishes were incubated with OPF (10 μmol/L) in culture medium for 24 h, washed with PBS twice and cultured with Dox-TCO (20 μmol/L) in DMEM medium for another 4 h. subsequently, free Dox-TCO was removed by replacing it with fresh culture medium and further incubated for 4h, 20 h, and 36(32?) h. CLSM images were taken at each operating steps to record the intracellular behavior of OPF and Dox-TCO, and line analysis was conducted to confirm their reaction in cell. Location of the anchored Dox-TCO after removal of the free Dox-TCO, and the distribution of free Dox-TCO was also investigated, which was conducted through co-localization analysis of Dox-TCO with Lyso-Tracker following the above described procedures. Dox-TCO was excited at 559 nm.

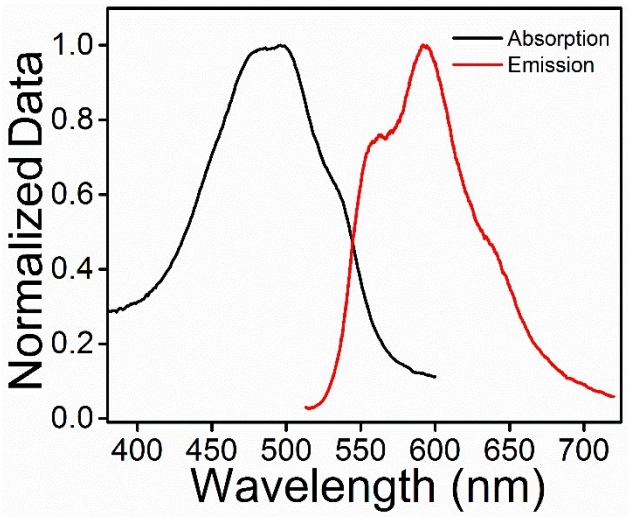


Figure S1. Normalized UV-vis absorption and fluorescence emission spectra of Dox-TCO with the excitation wavelength at 503 nm.

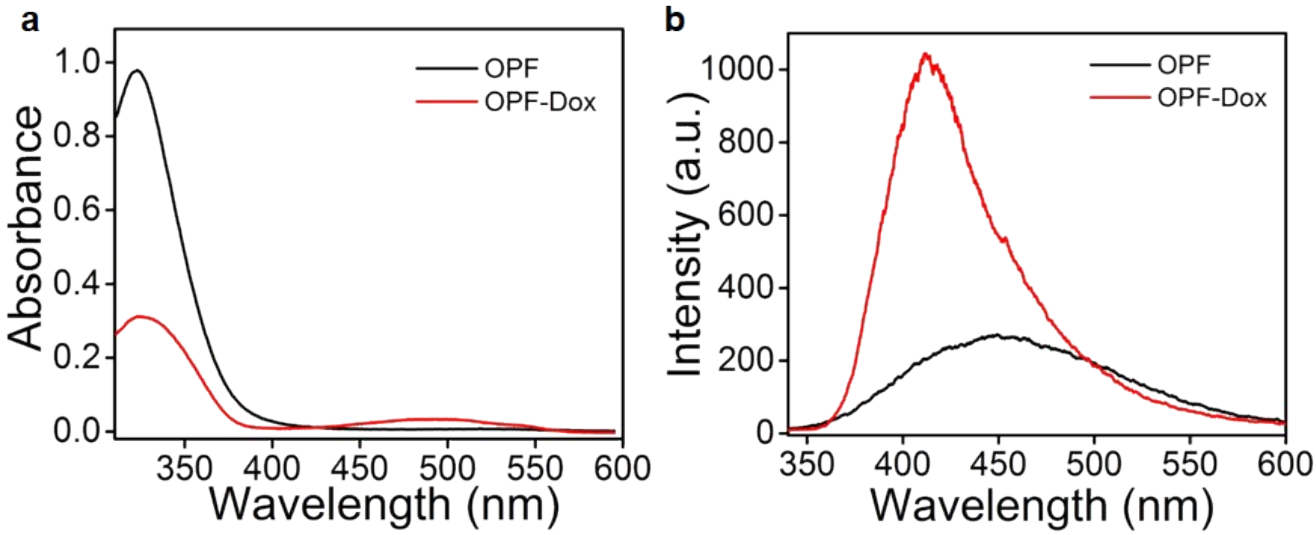


Figure S2. Absorption (a) and fluorescence emission (b) spectrum of OPF and OPF-Dox.

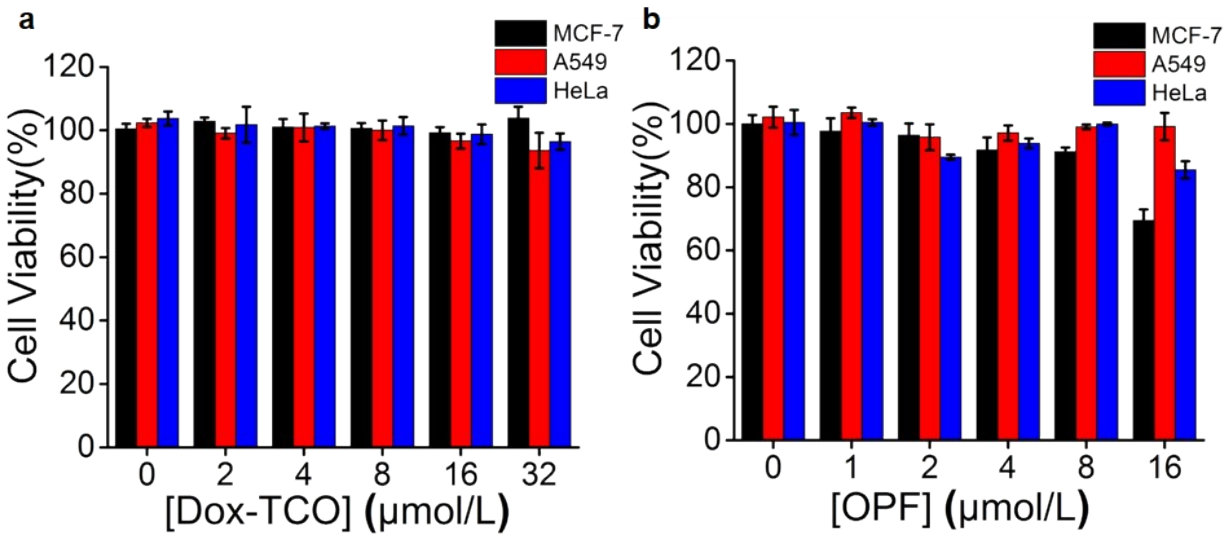


Figure S3. Cell viability of MCF-7 cells, A549 cells, and HeLa cells toward OPF (a) and Dox-TCO (b).

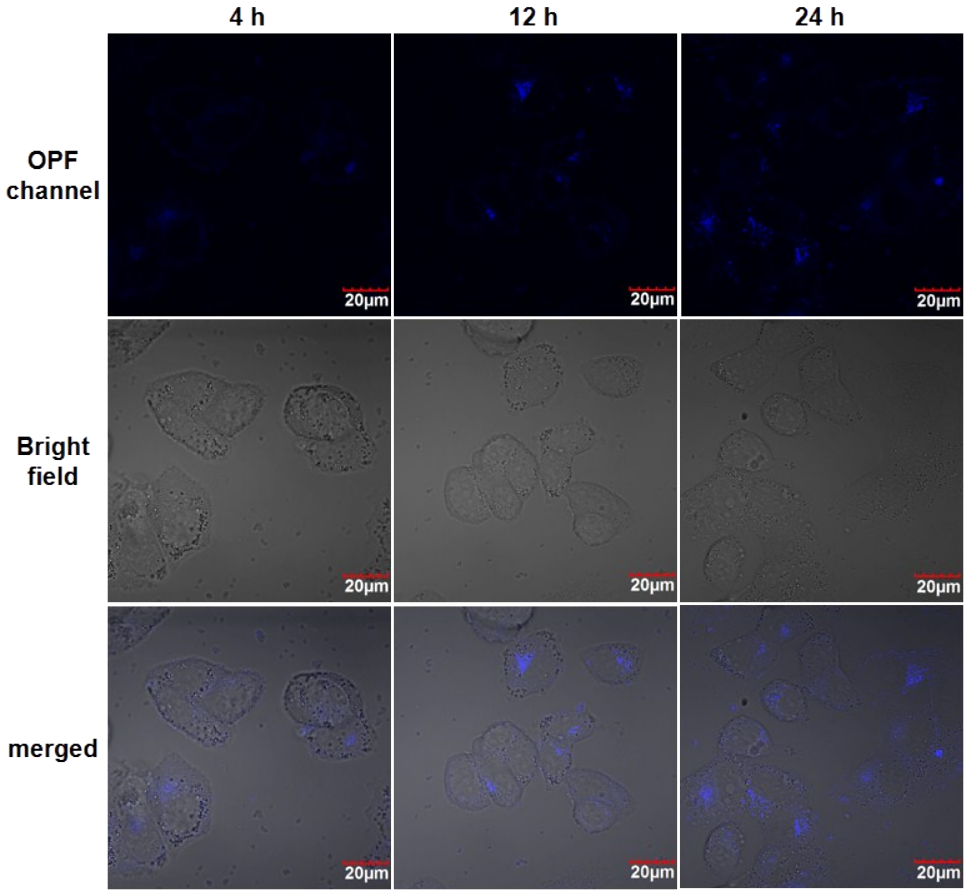


Figure S4. Fluorescence images of MCF-7 cells after incubated with OPF for different time.

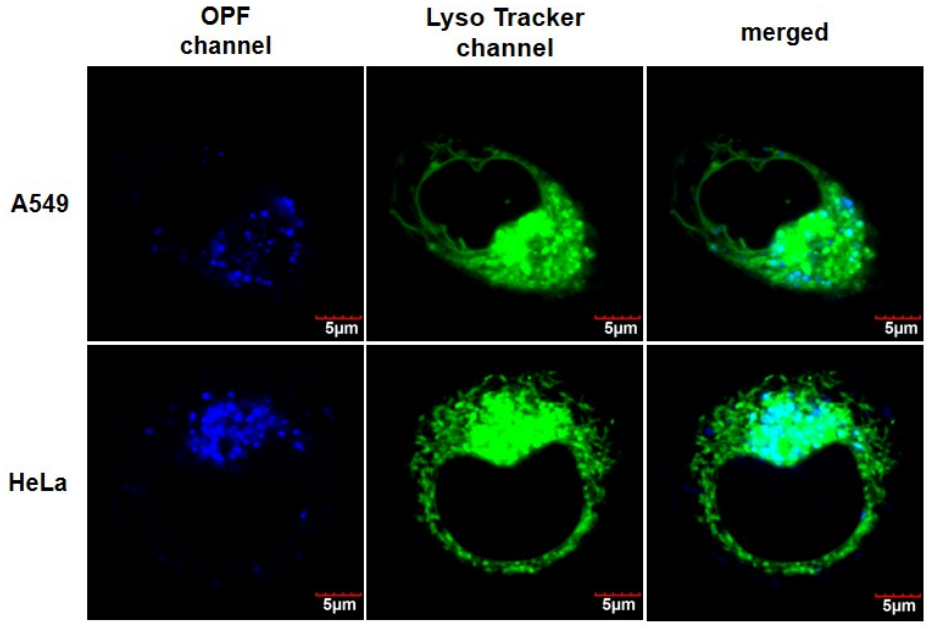


Figure S5. Fluorescence images of the intracellular localization of OPF in A549 cells and HeLa cells. Lyso-Tracker was used to analyze the colocalization of OPF.

References:

(1) Wieczorek, A.; Buckup,T.; Wombacher, R. *Org. Biomol. Chem.* **2014**, *12*, 4177.

(2) Xu, B. B.; Lu, M.; Kang, J. H.; Wang, D.; Brown, J.; Peng, Z. H. *Chem. Mater.* **2005**, *17,* 2841.