

## Supporting information

### **Fluorene-derived two-photon fluorescent probes for specific and simultaneous bioimaging of endoplasmic reticulum and lysosomes: group-effect and localization**

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## 1. Experimental Section

**Water solubility:** **TPFL-ER** and **TPFL-Lyso** were dissolved in DMSO to prepare stock solutions ( $1.0 \times 10^{-2}$  M), respectively. Then, the solution was diluted and added to a 1 cm standard quartz cell containing 3.0 ml of H<sub>2</sub>O using a micro syringe. In all cases, the concentration of DMSO in H<sub>2</sub>O was maintained to be 2.0 %. The absorbance was determined on HP-8453 spectrophotometer (Agilent, USA). Data were obtained from replicate experiments (n = 5). The obtained absorbance was analyzed by linear method. X-axis is the concentration of dyes in water and Y-axis is absorbance. When the absorbance deviates from the linear relationship, the corresponding maximum concentration is the solubility in water.

**Photostability in solution:** **TPFL-ER**, **TPFL-Lyso**, Lyso-tracker Red and ER-tracker Red were dissolved in DMSO-water (5:5 v/v) at a concentration of 2.0  $\mu$ M, respectively. The solutions were irradiated under a 500W iodine-tungsten lamp for 8 h at a distance of 250 mm away. An aqueous solution of sodium nitrite (50.0 g/L) as heat filter was placed between the samples and the lamp as a light filter (to cut off the light shorter than 400 nm). The photostabilities were expressed in the terms of remaining absorption (%) calculated from the changes of absorbance at the absorption maximum before and after irradiation by iodine-tungsten lamp. The absorbance was determined on HP-8453 spectrophotometer (Agilent, USA). Data were obtained from replicate experiments (n = 5).

**Preparation and staining of tissue sections:** Tissue sections were prepared from nude mice with S180 sarcoma. They were incubated with **TPFL-ER** and **TPFL-Lyso** (30  $\mu$ M) in artificial cerebrospinal fluid (ACSF) with 95% O<sub>2</sub> and 5% CO<sub>2</sub> for 30 min at 37 °C. Then washed three times with ACSF and transferred to glass bottom dish (MatTek, 35 mm dish with 20 mm bottom well).

**The spectral resolution (spectral separation technology):** Spectral resolution technology, that is spectral separation technology, is a kind of new technology appeared with the development of confocal, which has been widely applied in various kinds of confocal operating software. According to the spectral scanning or a fingerprint analysis, the height overlap spectrum of two dyes or probes, such as, FITC, GFP, FITC and Fluo3 highly overlapped within 500-530 nm, can be separated by using the spectral separation technology. In our work, the spectral separation technology was used for spectral separation after cells were simultaneously incubated with **TPFL-ER** (2.0  $\mu$ M) and **TPFL-Lyso** (2.0  $\mu$ M) to assess their cell staining efficiencies.

The colors in spectral resolution are pseudo colors. And adding pseudo color into cell figures is common method in cell imaging. There are no causal relationships between the pseudo color and emission wavelength region. Actually, the emission wavelength region of **TPFL-ER** is in the green fluorescence region, and that of **TPFL-Lyso** is in the kelly fluorescence region. So in this experiment, after cells were simultaneously incubated with **TPFL-ER** (2.0  $\mu$ M) and **TPFL-Lyso** (2.0  $\mu$ M), ER and Lyso images was separated with blue and green, respectively, to be easily distinguished by naked eye.

**Two-photon fluorescence imaging in tissues:** Two-photon fluorescence images of **TPFL-ER** and **TPFL-Lyso** in tissues were obtained with spectral confocal multiphoton microscopes (Olympus, FV1000) with a high-performance mode-locked titanium-sapphire laser source (MaiTai, Spectra-Physice, USA). Numerical aperture (NA) is 1.30 (sil). Excitation wavelength is at 750 nm. The images of **TPFL-ER** and **TPFL-Lyso** were recorded in the range of 510-570 nm. Internal PMTs were used to collect the signals in an 8 bit unsigned 1024×1024 pixels at 400 Hz scan speed. Data were obtained from replicate experiments (n = 5). Depth fluorescence imaging of **TPFL-ER** and **TPFL-Lyso** in tissues were obtained in the z-scan mode from 0  $\mu$ m to 500  $\mu$ m (step size = 3.0  $\mu$ m).

**Photostability in MCF-7 cells:** The fluorescence intensity in MCF-7 cells was determined staining with **TPFL-ER** (5.0  $\mu$ M) and **TPFL-Lyso** (5.0  $\mu$ M) for 30 min at 37 °C under 5% CO<sub>2</sub> by the spectral confocal multiphoton microscopes. The changes of fluorescence intensity in the consecutive t-scan mode were determined for 8h (time interval of data

determination = 60 min). Excitation wavelength = 750 nm/488nm. Emission wavelength = 510-570 nm. Data were obtained from replicate experiments (n = 5).

## 2. Synthetic procedures of TPFL-ER, TPFL-Lyso and intermediates

### Compound 2.

23.9 g 9, 9-dimethyl-nitrofluorene (**Compound 1**), 10 g calcium chloride and 210 g zinc were stirred and refluxed in ethanol (700 ml) for 8 h. After filtration, the residue was washed several times with boiling ethanol. The combined filtrates were poured into 2 L of water and white crystals were obtained as **Compound 2** (18.2 g, 89%).

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.58 (d,  $J = 7.5$  Hz, 1H), 7.51 (d,  $J = 8.0$  Hz, 1H), 7.36 (d,  $J = 7.4$  Hz, 1H), 7.31 - 7.23 (m, 1H), 7.21 (dd,  $J = 7.4, 0.9$  Hz, 1H), 6.76 (d,  $J = 2.1$  Hz, 1H), 6.67 (dd,  $J = 8.0, 2.1$  Hz, 1H), 1.44 (s, 6H). HRMS-ESI:  $m/z$  calcd.  $\text{M}^+$  for  $\text{C}_{15}\text{H}_{15}\text{N}$ , 209.1204; found, 209.1219.

### Compound 3 and Compound 4.

**Compound 2** (4.2 g, 20.0 mmol), iodomethane (1.6 mL, 20.0 mmol) and  $\text{K}_2\text{CO}_3$  (3.84 g, 22.0 mmol) were mixed in EtOH (100 mL), refluxed for 24 h under  $\text{N}_2$ . And then, the mixture was cooled to room temperature, poured into 50 mL of  $\text{H}_2\text{O}$ , and extracted with  $\text{CH}_2\text{Cl}_2$  for three times (25 mL  $\times$  3). The organic layer was dried with anhydrous  $\text{Na}_2\text{SO}_4$ . The crude product was purified by column chromatography on silica gel eluting with  $\text{CH}_2\text{Cl}_2$ /hexane (1:10, v/v) to provide **Compound 3** (1.51 g, 8 mmol, 40%) and **Compound 4** (1.69 g, 38%).

**Compound 3:**  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.57 (d,  $J = 7.5$  Hz, 1H), 7.54 (d,  $J = 8.2$  Hz, 1H), 7.36 (d,  $J = 7.4$  Hz, 1H), 7.27 (d,  $J = 1.1$  Hz, 1H), 7.20 (dd,  $J = 7.4, 1.0$  Hz, 1H), 6.69 (d,  $J = 2.1$  Hz, 1H), 6.62 (d,  $J = 8.2$  Hz, 1H), 2.92 (s, 3H), 1.45 (s, 6H). HRMS-ESI:  $m/z$  calcd.  $\text{M}^+$  for  $\text{C}_{16}\text{H}_{17}\text{N}$ , 223.1361; found, 223.1351.

**Compound 4:**  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.59 (d,  $J = 8.2$  Hz, 2H), 7.37 (d,  $J = 7.4$  Hz, 1H), 7.28 (td,  $J = 7.5, 1.2$  Hz, 1H), 7.19 (td,  $J = 7.4, 0.9$  Hz, 1H), 6.79 (s, 1H), 6.73 (d,  $J = 7.3$  Hz, 1H), 3.03 (s, 6H), 1.47 (s, 6H). HRMS-ESI:  $m/z$  calcd.  $\text{M}^+$  for  $\text{C}_{17}\text{H}_{19}\text{N}$ , 237.3395; found, 237.3391.

### Compound 5.

**Compound 3** (1.1 g, 5.0 mmol), 3-bromopropyne (0.55 mL, 7.5 mmol) and  $\text{K}_2\text{CO}_3$  (1.38 g, 10.0 mmol) was stirred in DMF (50 mL) at 90 °C for 24 h under  $\text{N}_2$ . Then, the mixture was cooled to room temperature, poured into  $\text{H}_2\text{O}$  (50 mL) and extracted with  $\text{CH}_2\text{Cl}_2$  for three times (25 mL  $\times$  3). The organic layer was dried with anhydrous  $\text{Na}_2\text{SO}_4$ . After removal of the solvent, the crude product was purified by column chromatography on silica gel eluting with  $\text{CH}_2\text{Cl}_2$ /hexane (1:15, v/v) to provide a white powder (**Compound 5**, 0.975 g, 3.75 mmol, 75%).

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.56 (d,  $J = 7.5$  Hz, 1H), 7.52 (d,  $J = 8.2$  Hz, 1H), 7.34 (d,  $J = 7.4$  Hz, 1H), 7.25 (d,  $J = 1.1$  Hz, 1H), 7.22 (dd,  $J = 7.4, 1.0$  Hz, 1H), 6.68 (d,  $J = 2.1$  Hz, 1H), 6.64 (d,  $J = 8.2$  Hz, 1H), 4.12 (d,  $J = 1.5$  Hz, 2H), 3.08 (s, 2H), 2.63 (s, 3H), 2.25 (s, 1H), 1.51 (s, 6H). HRMS-ESI:  $m/z$  calcd.  $\text{M}^+$  for  $\text{C}_{19}\text{H}_{19}\text{N}$ , 261.1517; found, 261.1509.

### Compound 6.

**Compound 5** (0.78 g, 2.6 mmol) and anhydrous aluminum chloride (1.6 g, 10.4 mmol) were added to 30 mL dry dichloromethane. Acetyl chloride (0.5 mL, 3.9 mmol) was added dropwise at 0 °C in 30 minutes. Then, the mixture was stirred for 5 h and slowly poured into a strongly stirred saturated sodium bicarbonate solution (500 mL). After extracted twice with dichloromethane (100 mL  $\times$  2), the organic layer was dried with anhydrous  $\text{Na}_2\text{SO}_4$ . The crude product was purified by column chromatography on silica gel eluting with  $\text{CH}_2\text{Cl}_2$ /hexane (1:1, v/v) to provide a yellow powder (**Compound 6**, 0.468 g, 52%).

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.00 (s, 1H), 7.91 (s, 1H), 7.64 (dd,  $J = 14.9, 8.1$  Hz, 2H), 6.95–6.81 (m, 2H), 4.14 (d,  $J = 1.5$  Hz, 2H), 3.09 (s, 2H), 2.64 (s, 3H), 2.23 (s, 1H), 1.50 (s, 6H). HRMS-ESI:  $m/z$  calcd.  $\text{M}^+$  for  $\text{C}_{21}\text{H}_{21}\text{NO}$ , 303.1627; found, 303.1635.

### Compound 8.

**Compound 7** was synthesized according to the reported reference <sup>7</sup>. NaI (5.1 g, 34.04 mmol), **Compound 7** (4.05 g, 34.04 mmol), morpholine (2.2 g, 25.85 mmol) and Na<sub>2</sub>CO<sub>3</sub> (3.6 g, 34.04 mmol) were dissolved in MeCN (80 mL) in room temperature and refluxed for 20 h. After filtration, the filtrate was concentrated and extracted with EtOAc for three times (80 mL × 3). The organic layers were combined, washed once with brine, dried with Na<sub>2</sub>SO<sub>4</sub>. Then, it was purified by column chromatography on silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/methanol (20:1, v/v) to provide the yellow oil (**Compound 8**, 4.42 g, 80%).

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 3.83 - 3.64 (m, 4H), 3.36 (t, J = 6.7 Hz, 2H), 2.52 - 2.34 (m, 6H), 1.77 (p, J = 6.8 Hz, 2H).

### TPFL-Lyso.

**Compound 6** (152 mg, 0.5 mmol), **compound 8** (133 mg, 0.5 mmol), CuSO<sub>4</sub>·5H<sub>2</sub>O (5 mol%, 6.2 mg, 0.025 mmol), and sodium ascorbate (10 mol %, 9.9 mg, 0.05 mmol) were added into the mixture solution of THF/H<sub>2</sub>O (7/3, 30 mL). The solution was stirred at room temperature overnight under N<sub>2</sub>. Then, the saturated NH<sub>4</sub>Cl (20 mL) was added to stop the reaction and THF was evaporated in vacuo. The residue was extracted with DCM for three times (20 mL × 3). The organic phase was dried with MgSO<sub>4</sub>. The crude product was purified by column chromatography on silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/methanol (200:1, v/v) to give a yellow solid (**TPFL-Lyso**, 122 mg, 51%).

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.98 (d, J = 1.1 Hz, 1H), 7.91 (dd, J = 7.9, 1.5 Hz, 1H), 7.60 (dd, J = 8.1, 5.3 Hz, 2H), 7.32 (s, 1H), 6.80 (dd, J = 9.6, 5.2 Hz, 2H), 4.75 (s, 2H), 4.37 (t, J = 6.8 Hz, 2H), 3.64 – 3.53 (m, 4H), 3.13 (s, 3H), 2.63 (s, 3H), 2.27 (s, 4H), 2.20 (s, 2H), 2.00 (s, 2H), 1.77 (s, 1H), 1.46 (s, 6H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 197.87, 156.94, 152.92, 149.84, 144.76, 134.35, 128.60, 127.14, 122.05, 121.98, 118.11, 111.94, 106.57, 66.85, 54.64, 53.38, 48.83, 47.87, 46.98, 39.08, 27.35, 26.83, 26.70. HRMS-ESI: m/z calcd. M<sup>+</sup> for C<sub>28</sub>H<sub>35</sub>N<sub>5</sub>O<sub>2</sub>, 473.2791; found, 473.2766.

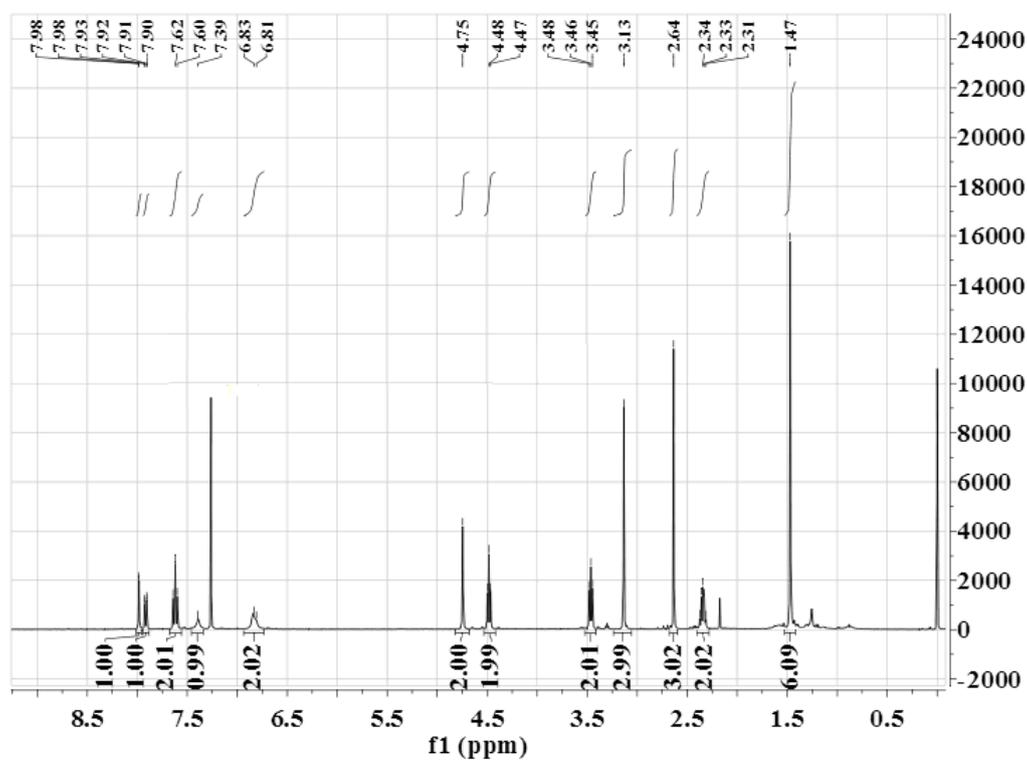
### TPFL-ER.

**Compound 6** (152 mg, 0.5 mmol), **compound 7** (133 mg, 0.5 mmol), CuSO<sub>4</sub>·5H<sub>2</sub>O (5 mol, 6.2 mg, 0.025 mmol), and sodium ascorbate (10 mol, 9.9 mg, 0.05 mmol) were added into the mixture solution of THF/H<sub>2</sub>O (5/5, 30 mL). The solution was stirred at room temperature overnight under N<sub>2</sub>. Then, the saturated NH<sub>4</sub>Cl (20 mL) was added to stop the reaction and THF was evaporated in vacuo. The residue was extracted with DCM for three times (20 mL × 3). The organic phase was dried with MgSO<sub>4</sub>. The crude product was purified by column chromatography on silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/methanol (200:1, v/v) to give a yellow solid (**TPFL-ER**, 107 mg, 0.22 mmol, 45%).

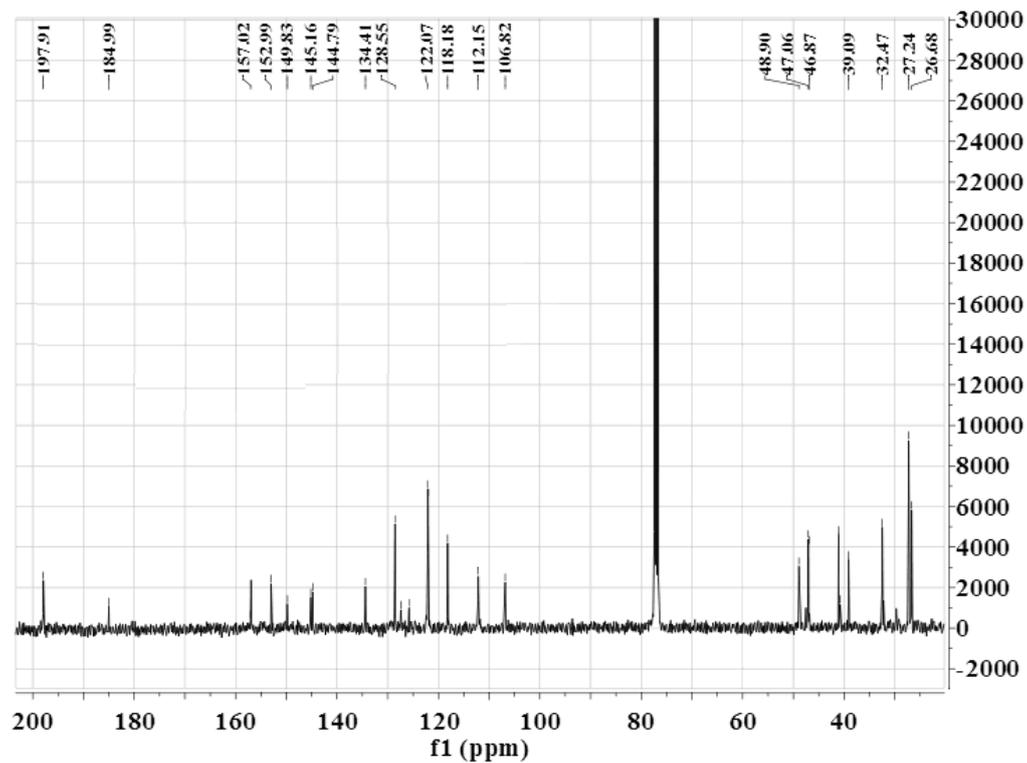
<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.98 (d, J = 1.2 Hz, 1H), 7.91 (dd, J = 8.0, 1.5 Hz, 1H), 7.62 (t, J = 8.3 Hz, 2H), 7.39 (s, 1H), 6.82 (d, J = 10.4 Hz, 2H), 4.75 (s, 2H), 4.48 (t, J = 6.6 Hz, 2H), 3.46 (t, J = 6.1 Hz, 2H), 3.13 (s, 3H), 2.64 (s, 3H), 2.40 – 2.28 (m, 2H), 1.47 (s, 6H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 197.91, 184.99, 157.02, 152.99, 149.83, 145.16, 144.79, 134.41, 128.55, 127.42, 125.79, 122.15, 122.07, 122.04, 118.18, 112.15, 106.82, 48.90, 47.06, 46.87, 41.08, 40.76, 39.09, 32.47, 27.24, 26.68. HRMS-ESI: m/z calcd. M<sup>+</sup> for C<sub>24</sub>H<sub>27</sub>N<sub>4</sub>O, 422.1718; found, 422.1727.

### 3. $^1\text{H}$ and $^{13}\text{C}$ NMR spectra of TPFL-ER and TPFL-Lyso.

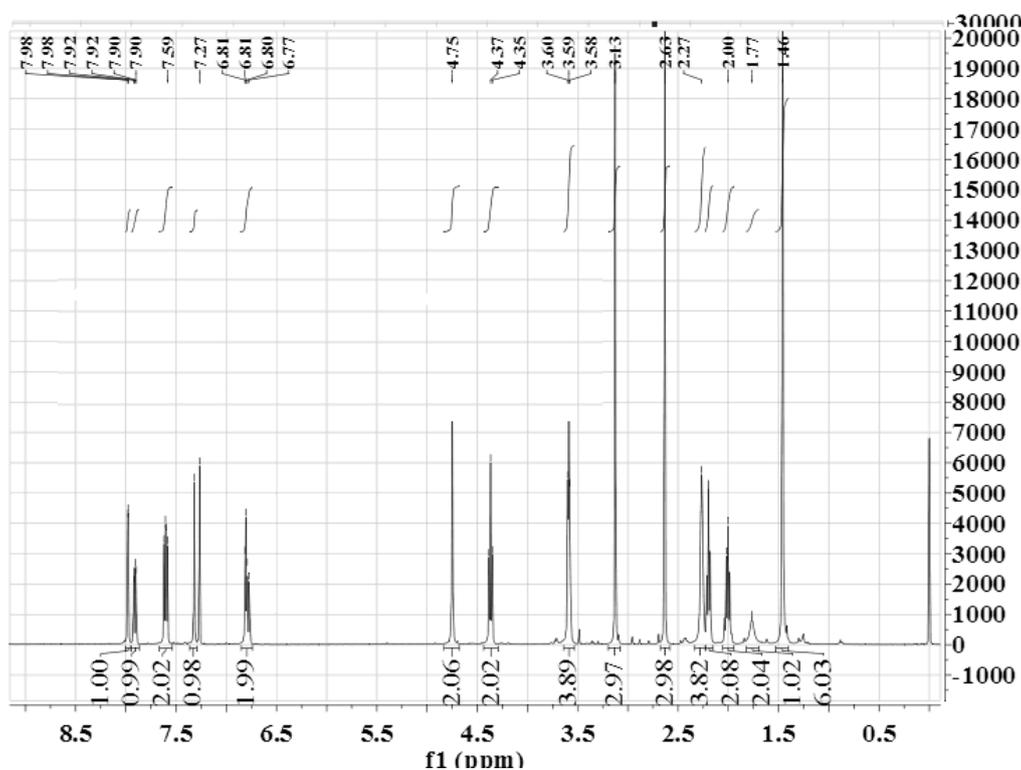
**a**



**b**



c



d

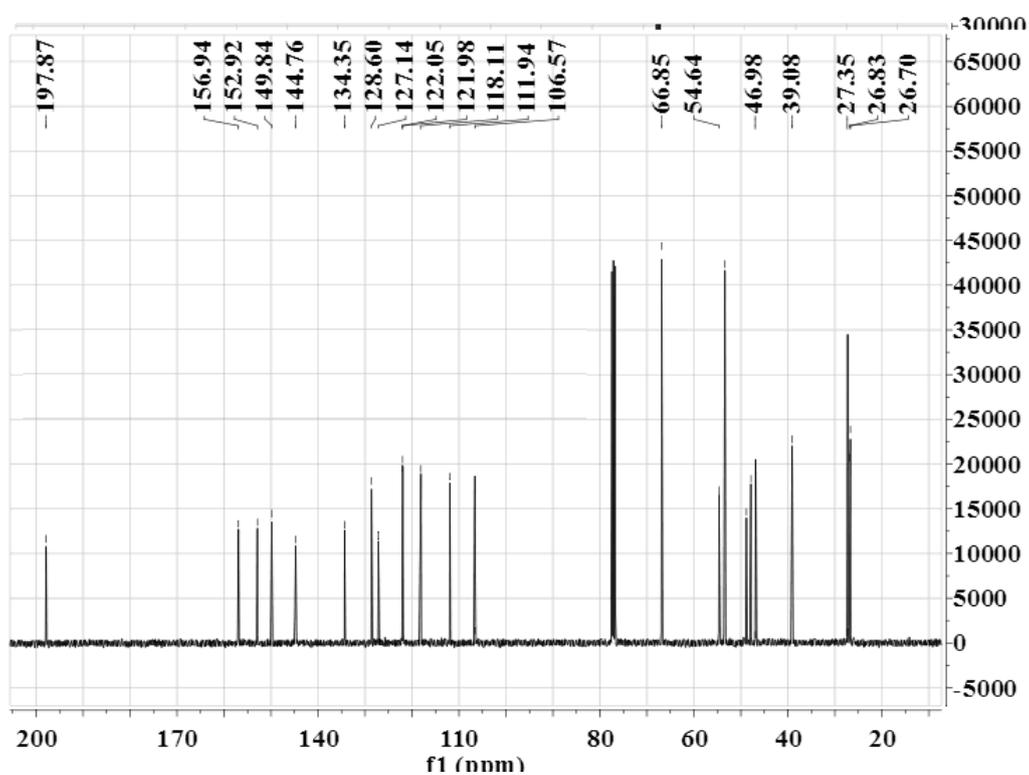


Figure S1. <sup>1</sup>H and <sup>13</sup>C NMR spectra of TPFL-ER (a, <sup>1</sup>H NMR and b, <sup>13</sup>C NMR) and TPFL-Lyso (c, <sup>1</sup>H NMR and d, <sup>13</sup>C NMR)

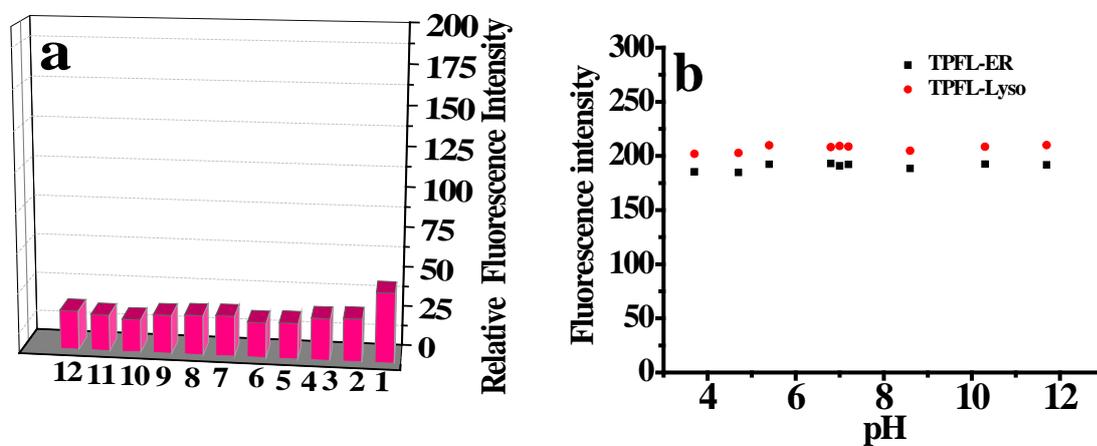
#### 4. Spectral data of TPFL, TPFL-ER and TPFL-Lyso dyes.

Table S1. Spectral data of TPFL, TPFL-ER and TPFL-Lyso dyes

Dyes	solvents	$\lambda_{\text{abs}}/\text{nm}$	$\lambda_{\text{em}}/\text{nm}$	$\Delta\lambda/\text{nm}$	$\Phi$	$\varepsilon \times 10^5/\text{M}^{-1}\text{cm}^{-1}$
<b>TPFL</b>	Dichloromethane	465	488	124	0.57	0.353
	PBS Buffer	467	570	215	0.06	0.201
<b>TPFL-ER</b>	Dichloromethane	477	521	144	0.65	0.389
	PBS Buffer	476	572	206	0.04	0.114
<b>TPFL-Lyso</b>	Dichloromethane	477	542	165	0.65	0.412
	PBS Buffer	476	570	209	0.03	0.106

As shown in Table S1, the fluorescence quantum yields ( $\Phi_{\text{TPFL-ER}}^{\text{buffer}} = 0.04$ ,  $\Phi_{\text{TPFL-Lyso}}^{\text{buffer}} = 0.03$ ) of the two probes in PBS buffer (pH 7.4) were much lower than those in hydrophobic dichloromethane ( $\Phi_{\text{TPFL-ER}}^{\text{buffer}} = 0.65$ ,  $\Phi_{\text{TPFL-Lyso}}^{\text{buffer}} = 0.65$ ). These results indicated that the strong fluorescence of **TPFL-ER** and **TPFL-Lyso** could only be observed in hydrophobic environments. Because that there is a specific interaction between water molecules and the structures of the probes that strongly perturbs the photophysical behavior of probes, which is expected by the classical Lippert-Mataga's theory of solvatochromism. However, dichloromethane is classic hydrophobic solvent, there is no interaction between water and the probes, so the QYs of two probes in PBS buffer much lower than that in dichloromethane in dichloromethane.

## 5. Selectivity and pH sensitivity of TPFL-ER and TPFL-Lyso.



**Figure S2.** (a) Changes in fluorescence with different hydrophobic environment in Tris-HCl buffer. 1. Control; 2. DNA; 3. RNA; 4. diastase; 5. lipase; 6. proteinase k; 7. histone; 8. collagen; 9. BSA; 10. hemoglobin; 11. trypsin; 12. chymotrypsin. (b) The fluorescence intensities of TPFL-ER (black) and TPFL-Lyso (red) in water as a function of pH (3.7-11.7).

## 6. Water solubility.

A small amount of **TPFL-ER** and **TPFL-Lyso** were dissolved in DMSO to prepare the stock solution ( $1.0 \times 10^{-2}$  M). The solution was diluted to ( $6.0 \times 10^{-3}$  -  $6.0 \times 10^{-5}$  M) and added to a 1 cm standard quartz cell containing 3.0 mL of H<sub>2</sub>O by using a micro syringe. In all cases, the concentration of DMSO in universal buffer was maintained to be 0.2 %. The absorbance of difference concentration **TPFL-ER** and **TPFL-Lyso** was determined on HP-8453 spectrophotometer (Agilent, USA). The obtained absorbance was analyzed by linear correlation method. X-axis is the concentration of dyes in water and Y-axis is absorbance. The plot of fluorescence intensity against the dye concentration was linear at low concentration and showed downward curvature at higher concentration (**Figure S3**). When the absorbance deviates from the linear relationship, the corresponding maximum concentration is the solubility of probe in water. So there is probably no nano-precipitation process before the concentration of dyes in water reach to the solubility.

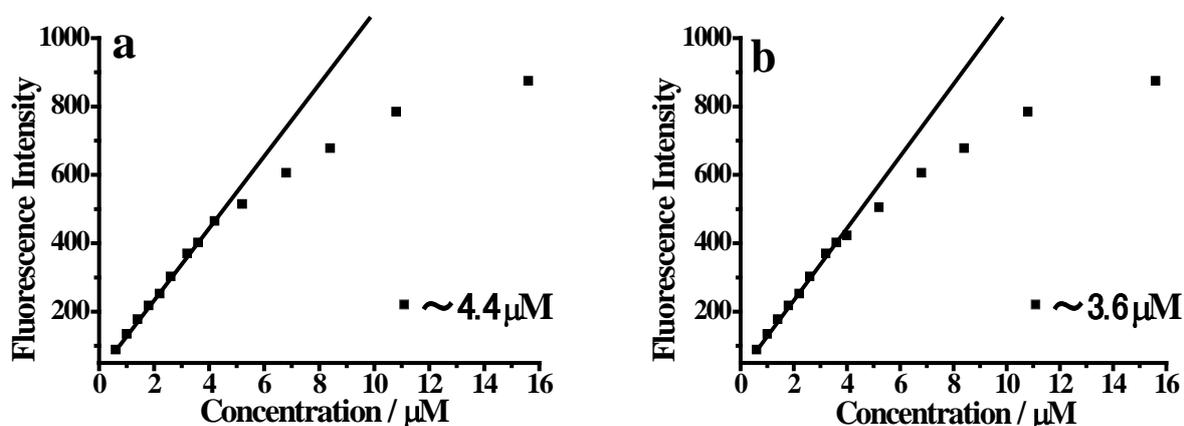
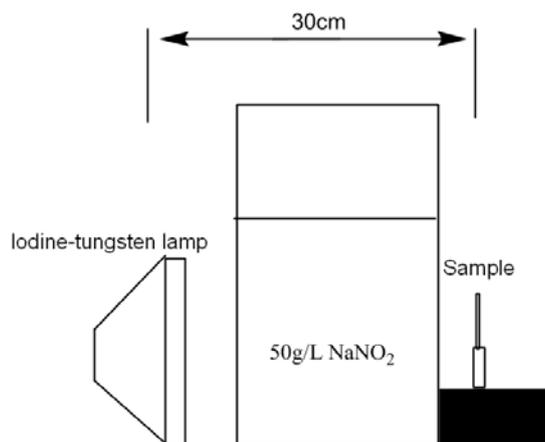


Figure S3. The plot of fluorescence intensity against dye concentration for TPFL-ER (a) and TPFL-Lyso (b) in water.

## 7. Photostability of TPFL-ER and TPFL-Lyso in MCF-7 cells and in solution.

TPFL-ER, TPFL-Lyso, Lyso-tracker Red and ER-tracker Red were dissolved in DMSO-water (5:5 v/v) at a concentration of 2.0  $\mu\text{M}$ , respectively. The solutions were irradiated under a 500W iodine-tungsten lamp for 8 h at a distance of 250 mm away. An aqueous solution of sodium nitrite (50.0 g/L) as heat filter was placed between the samples and the lamp as a light filter (to cut off the light shorter than 400 nm). The equipment was as follow:



The equipment of photostability measurement.

The photostabilities were expressed in the terms of remaining absorption (%) calculated from the changes of absorbance at the absorption maximum before and after irradiation by iodine-tungsten lamp. The absorbance was determined on HP-8453 spectrophotometer (Agilent, USA).

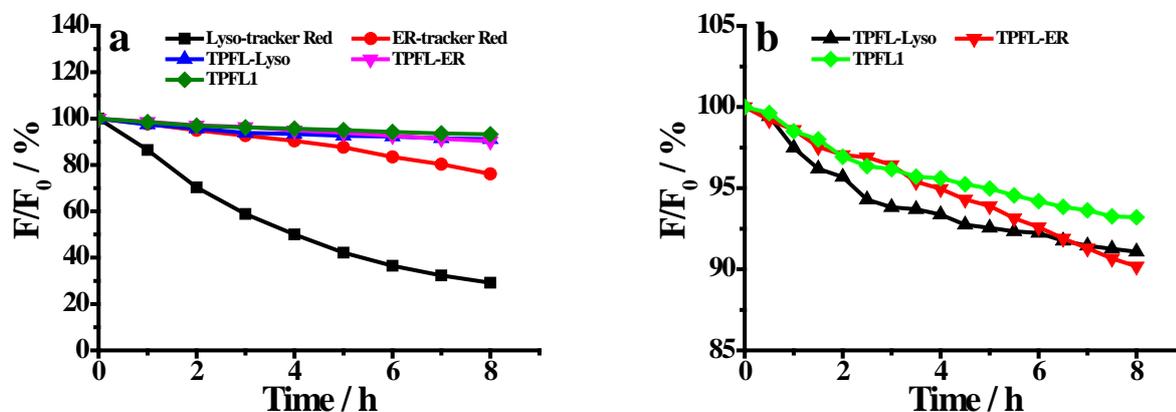


Figure S4. Photostabilities of TPFL-ER and TPFL-Lyso in MCF-7 cells (a) and in solution (b).

## 8. The fluorescence image in live-cells staining with TPFL-Lyso and TPFL-ER.

The selectivity of the two probes for ER and Lyso: In our molecular design, a chlorine group and a morpholine group was introduced into the structure template TPFL to form probes, TPFL-ER and TPFL-Lyso, respectively. As morpholine is a common lysosomal localization group, TPFL-Lyso targets at lysosomes. And the chlorine group was firstly expected to enable TPFL-ER to bind the chlorine pump of ER, which is a key target used in the specific localization of ER.

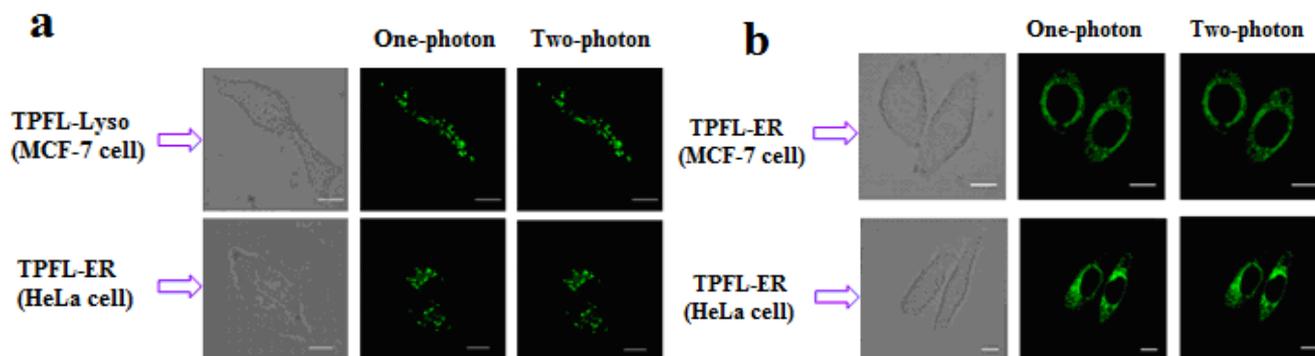


Figure S5. Fluorescence image in live-cells (MCF-7 and HeLa cells) staining with TPFL-Lyso (a) and TPFL-ER

(b).

## 9. Cytotoxicity by MTT method.

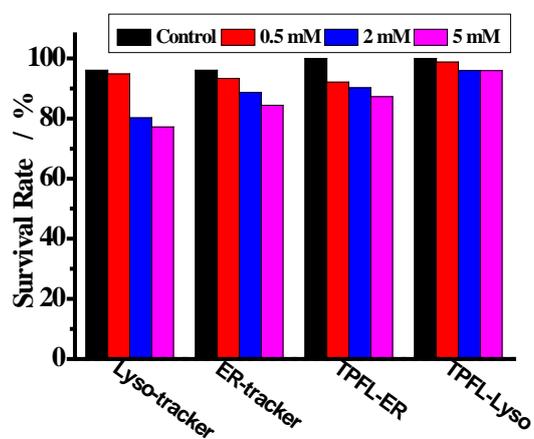
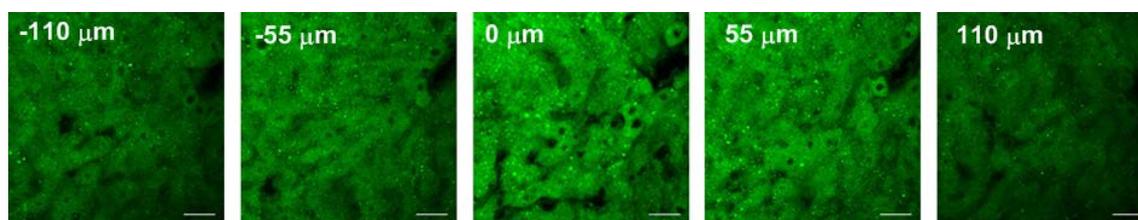


Figure S6. Cell toxicity in MCF-7 cells.

## 10. Tissue imaging.



**Figure S7. Imaging in tissue.** TPM images a fresh rat liver slice co-stained with 2.0 μM TPFL-Lyso and TPFL-ER. Fluorescence images were taken at depths of -110-110 μm. Scale bar, 30 μm.