Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Near-Infrared-Emitting Fluorescent Probe for Monitoring Mitochondrial pH

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General Materials and Instruments

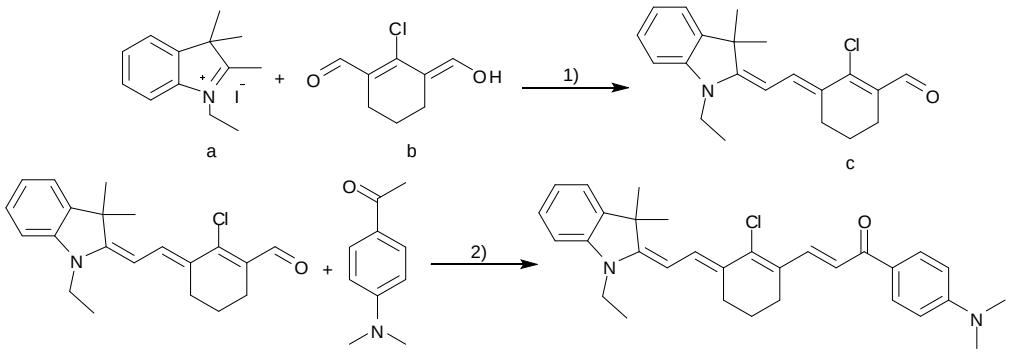
All reagents and solvents for reactions were used as received with the following exceptions. 1, 2-Dichloromethane (CH_2Cl_2), methanol (CH_3OH), Dimethyl Formamide (DMF) and triethylamine (Et_3N) were distilled from calcium hydride (CaH_2). L-buthionine-(S, R)-sulfoximine (BSO) was purchased from Sigma-Aldrich. Mito-Tracker Green and MitoSOX Red was purchased from Molecular Probes (Invitrogen, USA). Universal buffer media (0.1 M citric acid, 0.1 M KH_2PO_4, 0.1 M $Na_2B_4O_7$, 0.1 M Tris, 0.1 M KCl) was adjusted to corresponding pHs using 37% HCl solution and saturated NaOH solution. The Kunming (KM) mice were obtained from Shandong University Laboratory Animal Center. The experiments were approved by the institutional committee. All the animal experiments were carried out in accordance with the relevant laws and guidelines issued by the Ethical Committee of Shandong University.

Nuclear magnetic resonance (NMR) spectra were recorded mainly on a Bruker Avance II-400 Fourier Transform Spectrometer operating at 300 MHz for ^{1}H NMR and at 150 MHz for ^{13}C NMR. Mass spectra were recorded on a Bruker TOF-MS for high-resolution mass spectra. Fluorescence spectra measurements were performed using FLS-920 Edinburgh fluorescence spectrometer. The slit width was 3.5 nm for both excitation and emission. Samples were contained in 1.0 cm path length quartz cuvettes (5.0 ml volume). Cary Eclipse Fluorescence Spectrophotometer was used for the Kinetic assays. A Leica TCS SP5 system was used for confocal fluorescence imaging. The laser power of confocal imaging is 15 mW (488 nm laser) and 5 mW (543 nm laser), respectively. In vivo imaging was performed on IVIS Lumina III system with a metal halide lamp (150 W).

Synthesis

A condensation between the quaternary salt **a** and dialdehyde **b**, in butan-1-ol/benzene (7/3, v/v), is accomplished under reflux.[1,2] Then the resulting intermediate **c** reacted with a molar

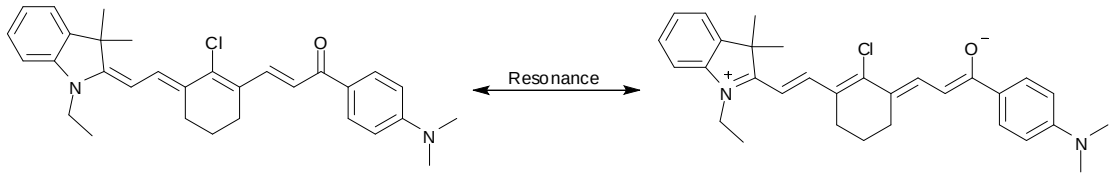
equivalent of 4'-dimethylaminoacetophenone, in the presence of alkali, to produce Spring Red (Scheme S1).



Conditions: (1) butan-1-ol/benzene (7/3, v/v), pyridine reflux, (2) KOH, reflux.

Scheme S1 The synthesis of Spring red

Intermediate c (0.341 g, 1 mmol) and 4'-dimethylaminoacetophenone (0.163 g, 1 mmol) were dissolved in KOH-methanol solution (5/15 mL). After the mixture was stirred over night at 80 °C under nitrogen, it was concentrated in vacuum. The residue was purified by column chromatography of silica gel with methanol/dichloromethane (1:50) as eluent and purple red product was obtained (yield 59%). ^{1}H NMR (300 MHz, DMSO): 1.15 (t, J = 7.2 Hz, 3H,), 1.57 (s, 6H), 1.79 (t, J = 5.4 Hz, 2H), 2.60 (m, 4H), 3.04 (s, 6H), 3.89 (q, J = 7.2 Hz, 2H), 5.56 (d, J = 12.6 Hz, 1H), 6.73 (d, J = 9.3 Hz, 1H), 6.76 (d, J = 9.0 Hz, 2H), 7.18 (m, 2H), 7.29 (d, J = 6.6 Hz, 1H), 7.53 (d, J = 12.6 Hz, 1H), 7.81 (d, J = 9.3 Hz, 1H), 7.97 (d, J = 9.0 Hz, 2H), 8.03 (d, J = 15.3 Hz, 1H) ^{13}C NMR (150 MHz, $CDCl_3$): 11.1, 21.5, 26.3, 27.4, 28.2, 29.7, 36.9, 40.1, 46.1, 92.6, 106.2, 110.6, 110.9, 120.1, 120.5, 121.8, 124.5, 127.5, 127.7, 127.9, 130.5, 130.6, 139.3, 139.8, 141.2, 143.9, 152.9, 158.8, 188.1. HRMS: $[M+H]^+$, calcd: m/z= 487.2511, found: m/z= 487.2480.



Scheme S2 Resonance structures of Spring Red

Computational study

The electron density of the HOMO level before protonation is distributed over the molecular skeleton and the nitrogen atom of dimethylamino group. However, after protonation of nitrogen atom, the coordination number of nitrogen changes from three to four, and nitrogen atom of dimethylamino group isn't involved in conjugation, which leading to decline of conjugation degree.

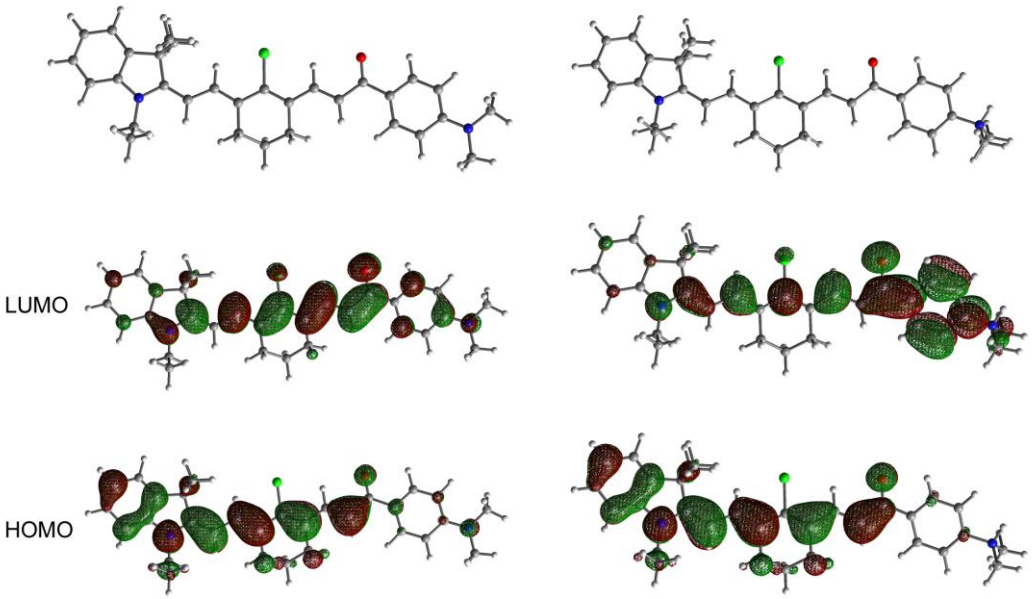


Fig. S1 DFT calculations of Spring Red before and after protonation. The calculations were carried out using Gaussian 09. The two molecules were optimized in the gas phase at B3LYP/6-31G(d) level. Harmonic frequency analysis calculations were performed at the same level to verify the optimized structures to be minima.

Spectral properties of Spring Red

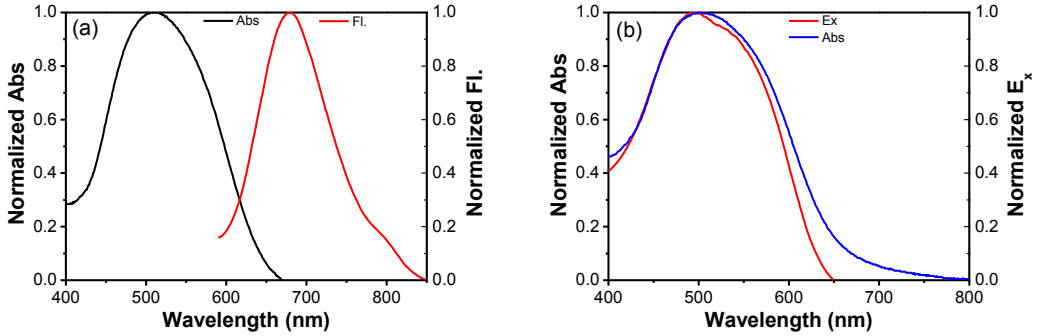


Fig. S2 (a) Absorption and fluorescence spectra of Spring Red (Abs: λ_{max}=529 nm, 10 μM in universal buffer, 10 mM, pH=8.0, with a molar extinction coefficient (ε) 3.0×10^4 M^{-1}·cm^{-1}; Fluo: 10 μM in universal buffer, 10 mM, pH=8.0, with fluorescence quantum yield 1.0% using Rhodamine B in ethanol (Φ_f =0.89) as the reference.[3,4] (b) Absorption (λ_{max}=501 nm) and excitation spectra of Spring Red upon protonation (10 μM, in universal buffer,10 mM, pH=2.2).

Interference experiment of Spring Red

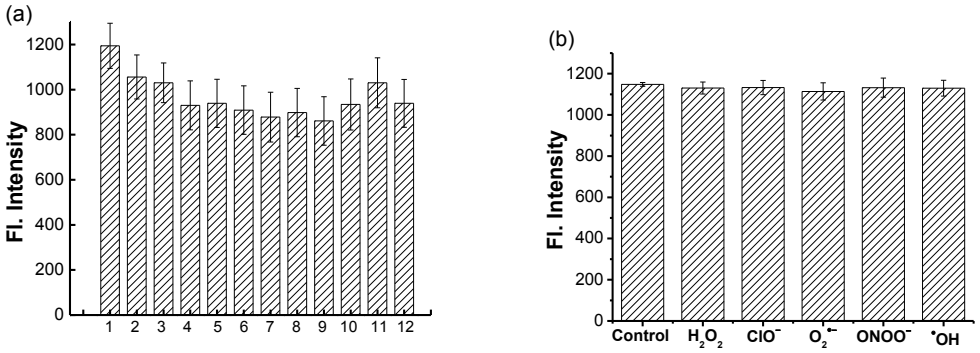


Fig. S3 Fluorescence responses of 30 μM Spring Red in universal buffer (10 mM pH 7.4) to diverse ions (a) and common ROS (b). 1: blank; 2: K^+ (12 mM); 3: Na^+ (12 mM); 4: Ca^{2+} (0.5 mM); 5: Mg^{2+} (0.5 mM); 6: Zn^{2+} (0.3 mM); 7: Cu^{2+} (0.3 mM); 8: Mn^{2+} (0.3 mM); 9: Co^{2+} (0.3 mM); 10: NH_4^+ (0.5 mM); 11: Cl^- (12 mM); 12: SO_4^{2-} (0.5 mM); H_2O_2: 10 mM; ClO^-, $O_2^{\bullet-}$, $ONOO^-$, $^{\bullet}OH$: 200 μM. $\lambda_{ex}/\lambda_{em}$ = 530/680 nm

Fluorescence imaging in zebrafish with Mito-Tracker Green

Zebrafish were incubated with Mito-Tracker Green (25 nM) for 15 min. Then, after anesthetized, fluorescence imaging of the zebrafish were fulfilled. The excitation wavelength

was 488 nm, and the emission was collected at 495-520 nm. Intense fluorescence was observed in the heart region, which verify the high concentration of mitochondria in the heart of zebrafish, and might further confirm the mitochondria-targeting capability of Spring Red.

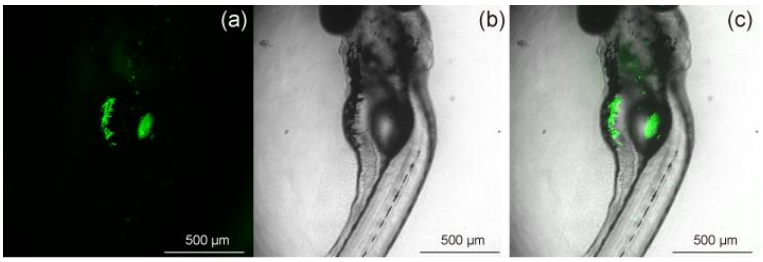


Fig. S4 Confocal fluorescence imaging of Mito-Tracker Green (25 nM) in zebrafish. (a) Fluorescence imaging between 495-520 nm, λ_{ex}=488 nm. (b) Brightfield. (c) Merged images of a and b.

Imaging experiments of analogous compounds

To further affirm the mitochondria-targeting capability of Spring Red, we synthesized three analogues of Spring Red by replacing dimethylamino with hydrogen, methyl and methoxyl group (Fig. S5A). Colocalization-imaging results showed that three compounds also possess mitochondria-targeted capability to some extent (Fig. S5B). So the main structures of the compounds also play an important role for targeting mitochondria. Both the dimethylamino group and lipophilic cation contribute to the mitochondria-targeting capability of Spring Red.

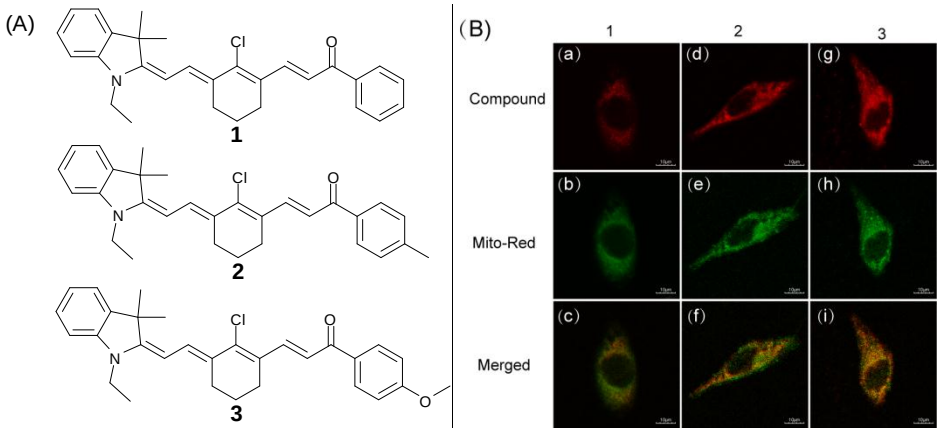


Fig. S5 (A) The structures of Spring Red analogues. (B) Colocalization-imaging experiments of synthesized compounds and MitoSOX Red.

Kinetic assays

The photostability of Spring Red was tested during 60 min. the result suggests the probe is quite stable to the medium, light and air.

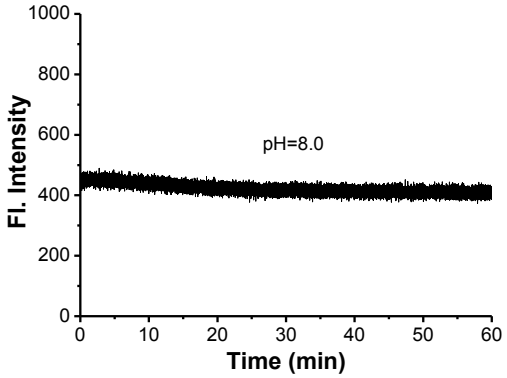


Fig. S6 The kinetic assay of Spring Red (30 μM). The emission spectra were recorded at 680 nm, in 10 mM universal buffer pH=8.0, and Cary Eclipse Fluorescence Spectrophotometer was used.

Colocalization-imaging experiments in HepG2 cells

HepG2 cells were passed and dispersed on 18 mm glass coverslips at 37 °C, 5% CO_2 1 day before imaging. Then cells were incubated with Mito-Tracker Green (25 nM) and Spring Red (30 μM) for 15 min. The medium was removed and cells were washed with PBS (10 mM, pH 7.4) for three times. Confocal images of cells fluorescence of the Mito-Tracker Green were captured using a 488 nm laser, the collection window is 495–520 nm. The excitation wavelength of Spring Red is 543 nm, and the collection window is 650–800 nm.

Test of photostability of Spring Red in live cells

We incubated live HL-7702 cells using Spring Red, for observing changes in fluorescence brightness within these cells during 30 min (Fig. S7). Three regions in visual field were selected (Fig. S8 a), and corresponding fluorescence intensity was obtained by confocal laser

scanning microscope respectively during 30 min (Fig. S8 b). Average fluorescent intensity within these cells maintained unaltered basically throughout the experiment. These data show there is no significant loss of intracellular dye over at least 30 min (Fig. S8). The results display that the dye has good biocompatibility, excellent membrane permeability, as well as good photostability.

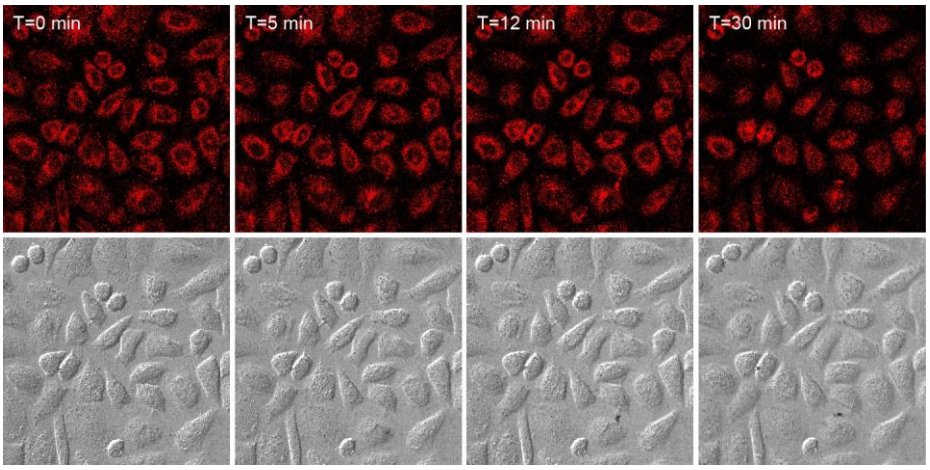


Fig. S7 Confocal fluorescence images of HL-7702 cells during 30 min. The cells were incubated with Spring Red 30 μM for 10 min at 37 °C and then washed with universal buffer at pH 7.4. Samples were excited with a 543 nm laser, and fluorescence was acquired in the range of 650-800 nm.

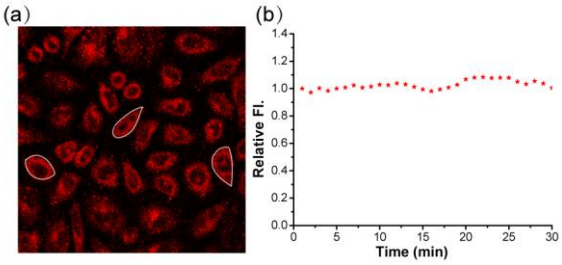


Fig. S8 (a) Photobleaching assay of Spring Red.30 μM Spring Red in HL-7702 cells, the excitation wavelength was recorded at 543 nm. (b) Average fluorescence intensity of three white circle regions.

Cytotoxicity assay of Spring Red

HL-7702 cells (1.0×10^5 cell ml^{-1}) were dispersed in replicate 96-well microtiter plates to a total volume of 200 μl $well^{-1}$. Plates were maintained at 37 °C in a 5% CO_2/95% air incubator for 12

h. The cells were incubated with different concentrations dye (1.0 ×10^{-4}, 1.0 ×10^{-5}, 1.0 ×10^{-6}, 1.0 ×10^{-7} and 1.0 ×10^{-8} M) for an additional 12 h. Subsequently, MTT solution (5.0 mg ml^{-1}, universal buffer) was added to each well. After 4 h, the remaining MTT solution was removed, and 150 μl of DMSO was added into each well, followed by further incubation for 4 h at 37 °C. Absorbance was measured at 530 nm in a TRITURUS microplate reader. IC50 values was calculated to be 4.8×10^{-3} M according to Huber and Koella.[5] The results show that Spring Red is low toxic throughout cell imaging experiments.

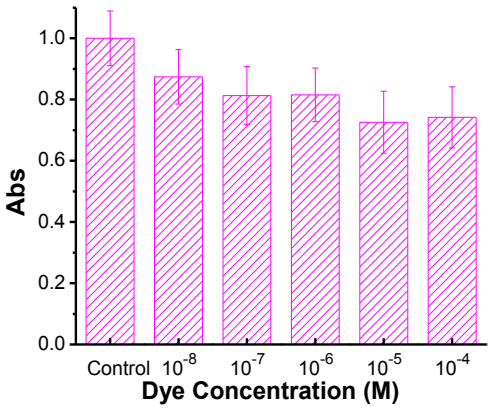


Fig. S9 MTT assay of live HL-7702 cells in the presence of Spring Red of various concentrations.

Intracellular pH calibration

The HepG2 cells were incubated with high K^+ buffer (30 mM NaCl, 120 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$,1 mM NaH_2PO_4, 5 mM glucose, 20 mM HEPES, and 20 mM NaOAc) at various pH values (4.0-8.5) in the presence of 10.0 μM of nigericin. After 30 min, Spring Red was added to cells and incubated for 10 min at 37 °C. The fluorescence images were captured, and the pH calibration curve was constructed with confocal microscope.

pH changes in BSO-treated HepG2 cells

The human hepatoma cells HepG2 undergo apoptosis upon treatment with L-buthionine-(S, R)-sulfoximine (BSO). The morphological changes of cells were well characterized by cell

shrinkage (arrow 1, 2 in Fig. S10), indicating the occurrence of apoptosis. With the aid of the probe, we observed pH variations in the process of cellular apoptosis, which decreased from 7.96 to 7.61. The visualization results are consistent with previous studies on molecular events during apoptosis.

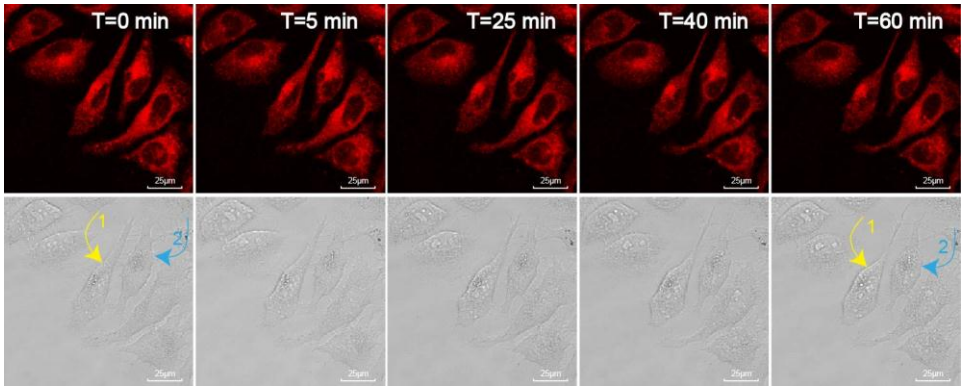


Fig. S10 Fluorescence imaging of pH changes during HepG2 apoptosis. HepG2 cells were loaded with Spring Red (30 μM) for 10 min, and washed away, then, BSO (5 mM) was added. The excitation wavelength was recorded at 543 nm, and the emission wavelength was collected at 650-800 nm.

In Vivo Fluorescence Imaging

KM mice (20−25 g) were given an intraperitoneal (i.p.) injection of LPS (1 mg in 400 μl saline). After 4 h, the mice were anesthetized by an i.p. injection of 4% chloral hydrate (0.25 ml). Then, the mice were intraperitoneally injected with Spring Red (50 μM). In addition, mice (only treated with LPS but no Spring Red and unstimulated mice intraperitoneally injected only with Spring Red (50 μM) were prepared. The mice were then imaged (10 min after the injection of Spring Red) by using a IVIS Lumina III in vivo imaging system, with an excitation filter of 530 nm and an emission filter of 670 nm.

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