Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

for

An excitation ratiometric Zn^{2+} sensor of mitochondria-targetability and its monitoring of mitochondrial Zn^{2+} releasing upon different stimulations

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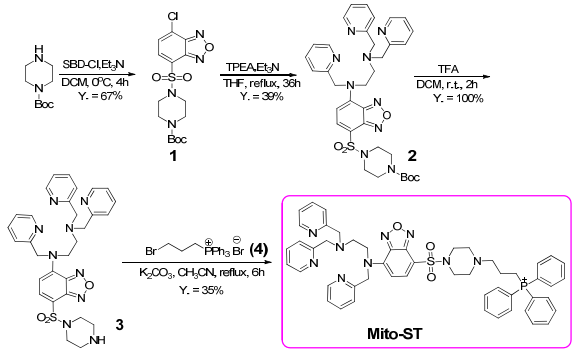
Contents:

S1. Materials and general methods

All the solvents were of analytic grade. The stock solutions of metal ions for fluorescence discrimination were prepared from $MnCl_2$, $PbCl_2$, $CoCl_2·6H_2O$, Zn $(NO_3)_2·7H_2O$, $CaCl_2$, NaCl, $CuSO_4$, $NiCl_2·6H_2O$, KCl, $CdCl_2·2.5H_2O$, $HgCl_2$, $MgCl_2·6H_2O$ using doubly distilled water. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker DRX-500 spectrometer with TMS as internal standard in $CDCl_3$. Mass spectrometric data were determined with a LCQ (ESI-MS，Thermo Finnigan) mass spectrometer. Fluorescence measurements were performed on an AMINCO Bowman series 2 Spectrofluorometer with 4 nm slit for both excitation and emission. Absorption spectra were measured on a Shimadzu UV-3100 or an UV-VIS-NIR spectrophotometer. All pH measurements were determined by a Model PHS-3C meter.

S2. Synthesis of Mito-ST



Scheme S1. Synthesis of Mito-ST.

Synthsis of compound 1 SBD-Cl (0.438 g, 1.53 mmol) and 8 mL CH_2Cl_2 were mixed in a 100 mL three-necked bottom round flask and the mixture was cooled to 0°C with stirring. Then, 10 mL CH_2Cl_2 containing *N*-but-piperazine (0.285 g, 1.53 mmol) was added slowly into the flask via a syringe over 1 h. The result mixture was sitrred at room temperature for an additional hour. After that, the aqueoues sloution was washed with brine three times and dried over $MgSO_4$. Then the solvent were removed by evaporation in vacuo and the crude product was purified by silica gel chromatography. Dicholormethane/ethyl acetate (v/v, 20: 1) was used as the eluent. Yield, 67%. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.44 (s, 9 H, -Bu^t), 3.34 (t, 4 H, J = 5.0 Hz, -CH_2CH_2-), 3.54 (t, 4 H, J = 5.0 Hz, -CH_2CH_2-), 7.57(d, 1 H, J = 10.0 Hz, BD-H), 7.98 (d, 1 H, J = 10.0 Hz, BD-H)

ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 28.17, 43.09, 45.76, 80.48, 125.82, 128.08, 129.06, 134.50, 145.39, 148.75, 153.99 ppm. ESI-MS: calculated for $[M]^+$: 402.08, found: 402.39.

Synthsis of compound 2 Compound **1** (0.214 g, 0.53 mmol), K_2CO_3 (0.088 g, 0.64 mmol), and TPEA (0.177 g, 0.53 mmol) were mixed in 10 mL of CH_3CN. The mixture was refluxed for 10 h with stirring. Then the solids were filtered off and washed with CH_2Cl_2. After combining the filtration and the CH_2Cl_2 solution, the solvents were removed by evaporation in vacuo. The hygroscopic product was obtained by purifying the resulting mixture with silica gel chromatography. Dicholormethane/methanol (v/v, 25: 2) was used as the eluent. Yield, 39%. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.43 (s, 9 H, -Bu^t), 3.13 (t, 2 H, J = 5.0 Hz, -CH_2CH_2-), 3.20 (t, 4 H, J = 5.0 Hz, -CH_2CH_2-), 3.50 (t, 4 H, J = 5.0 Hz, -CH_2CH_2-), 4.04 (s, 4 H, J = 5.0 Hz, -CH_2Py), 4.24 (t, 2 H, J = 5.0 Hz, -CH_2CH_2-), 5.15 (s, 2 H, -CH_2Py), 6.08 (d, 1 H, J = 10.0 Hz, BD-H), 7.18-7.71 (m, 10 H, BD-H and Py-H), 8.52 (d, 2 H, J = 5.0 Hz, Py-H), 8.56 (d, 1 H, J = 5.0 Hz, Py-H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 28.19, 43.11, 45.74, 50.49, 51.54, 58.27, 60.71, 80.22, 102.14, 107.62, 120.84, 122.18, 122.58, 123.13, 136.42, 136.89, 138.63, 142.14, 144.24, 146.89, 149.03, 149.77, 154.10, 155.95, 158.51 ppm. ESI-MS: calculated for $[M]^+$: 699.30, found: 699.47.

Synthsis of compound 3 Compound **3** (0.142 g, 0.20 mmol) and 2 mL CH_2Cl_2 were added into a 50 mL two-necked bottom round flask and cooled to 0^{o}C. Then, 2 mL CH_2Cl_2 contaning TFA (0.5 mL, excess) was slowly added into the flask via a syringe over 30 min. The result mixture was sitrred at room temperature for 2 hours. After that, the solvents were removed by evaporation in vacuo. The crude product was used directly for next step without further purification.^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.93 (t, 4 H, J = 5.0 Hz, -CH_2CH_2-), 3.02 (t, 2 H, J = 5.0 Hz, -CH_2CH_2-), 3.20 (t, 4 H, J = 5.0 Hz, -CH_2CH_2-), 4.02 (s, 4 H, J = 5.0 Hz, -CH_2Py), 4.13 (t, 2 H, J = 5.0 Hz, -CH_2CH_2-), 5.13 (s, 2 H, -CH_2Py), 6.01 (d, 1 H, J = 10.0 Hz, BD-H), 7.13-7.69 (m, 10 H, BD-H and Py-H), 8.50 (d, 2 H, J = 5.0 Hz, Py-H), 8.57 (d, 1 H, J = 5.0 Hz, Py-H) ppm.

Synthsis of Mito-ST Compound **3** (0.214 g, 0.20 mmol), K_2CO_3 (0.033 g, 0.24 mmol), and Compound **4** (0.092 g, 0.20 mmol) were mixed in 5 mL of CH_3CN. The mixture was refluxed for 6 h. Then the solids were filtered off, and the product was obtained by purifying the resulting mixture with silica gel chromatography. Dicholormethane/methanol (v/v, 15: 1, R_f = 0.20) was used as the eluent. Yield, 35%. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.77 (t, 2 H, J = 5.0 Hz, -CH_2CH_2-), 2.41 (t, 4 H, J = 5.0 Hz, -CH_2CH_2-),2.68 (t, 2 H, J = 5.0 Hz, -CH_2CH_2-), 3.02 (t, 2 H, J = 5.0 Hz, -CH_2CH_2-), 3.17 (t, 4 H, J = 5.0 Hz, -CH_2CH_2-), 3.92 (s, 4 H, J = 5.0 Hz, -CH_2Py), 3.97 (t, 2 H, J = 5.0 Hz, -CH_2CH_2-), 4.11 (t, 2 H, J = 5.0 Hz, -CH_2CH_2-), 5.10 (s, 2 H, -CH_2Py), 6.01 (d, 1 H, J = 10.0 Hz, BD-H), 7.13-7.21 (m, 4 H, Py-H), 7.41 (d, 2 H, J = 5.0 Hz, Py-H), 7.59-7.85 (m, 20 H, BD-H, Ar-H and Py-H), 8.48 (d, 2 H, J = 5.0 Hz, Py-H), 8.54 (d, 1 H, J = 5.0 Hz, Py-H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 20.08, 29.67, 45.75, 51.61, 52.20, 56.52, 56.66, 58.29, 60.86, 102.43, 108.10, 118.11, 118.79, 121.01, 122.30, 122.67, 123.26, 130.41, 130.50, 133.55, 133.63, 135.01, 136.58, 137.10, 138.83, 142.15, 144.46, 147.10, 149.09, 149.81, 156.07 ppm. ^{31}P NMR (500 MHz, $CDCl_3$): 25.09 ppm. ESI-MS: calculated for $[M]^+$: 902.37, found: 902.42. Element analysis (%): calcd for $C_{51}H_{53}N_9O_3PS$: C, 67.83; H, 5.92; N, 13.96; found: C, 67.64; H, 6.03; N, 14.12.

S3. NMR and MS spectra of Mito-ST

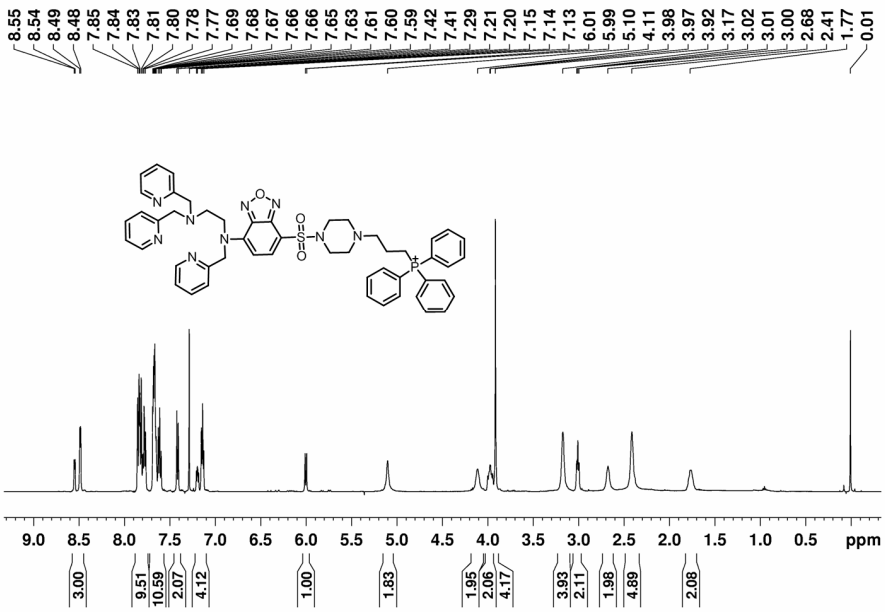


Figure S1. ^{1}H NMR spectrum of **Mito-ST** in $CDCl_3$.

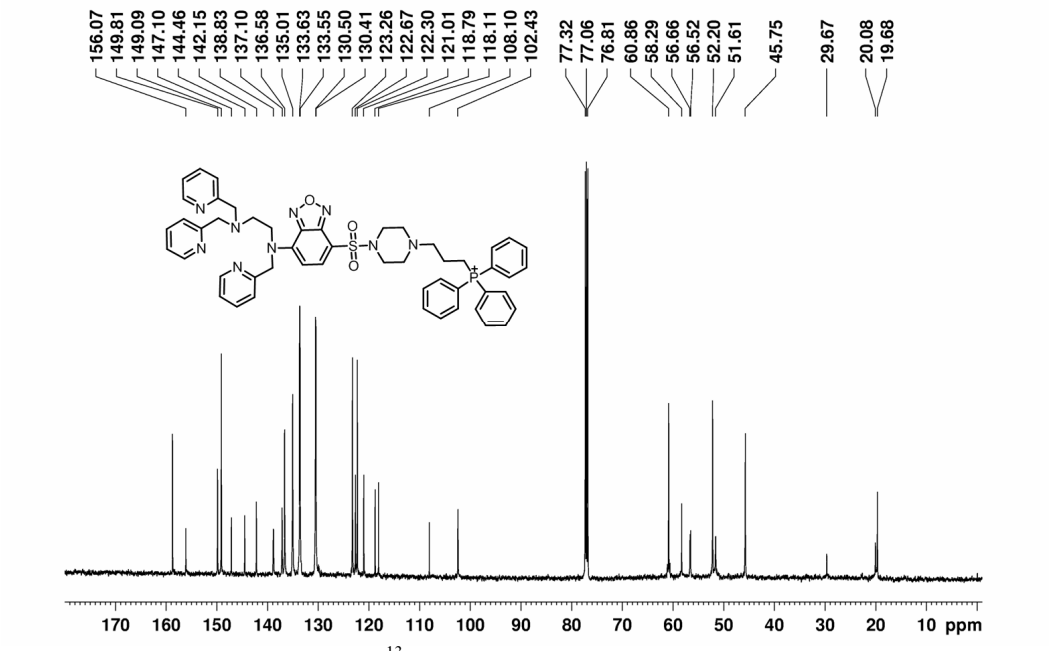


Figure S2. The ^{13}C NMR spectrum of **Mito-ST** in $CDCl_3$.

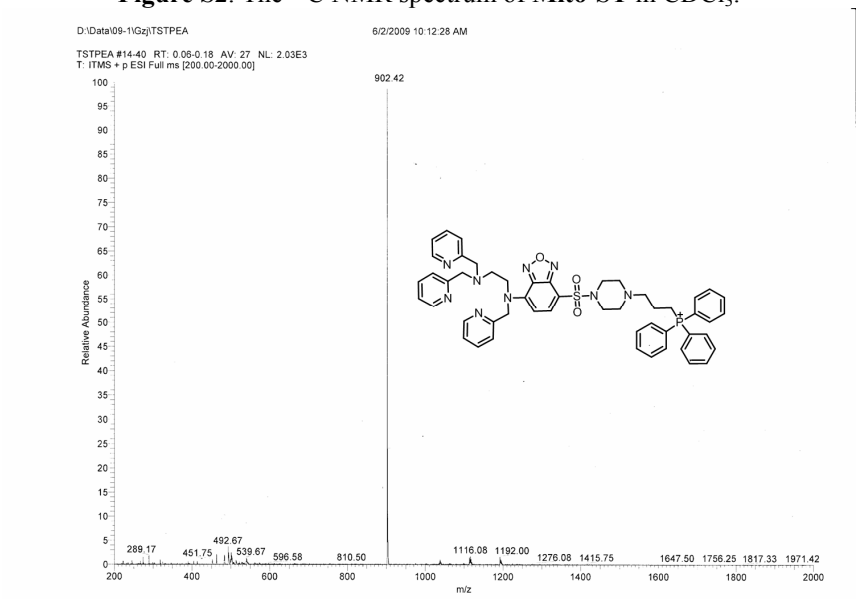


Figure S3. ESI-MS spectrum of **Mito-ST**.

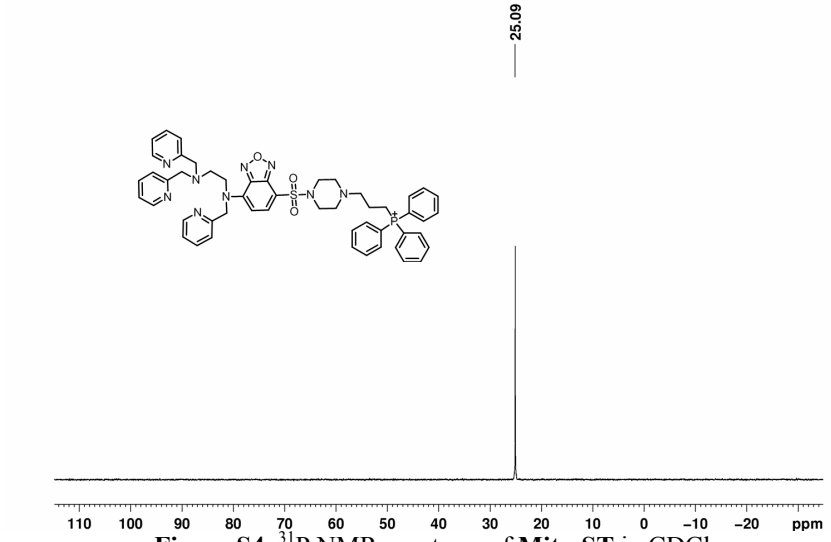


Figure S4. ^{31}P NMR spectrum of **Mito-ST** in $CDCl_3$.

S4. Absorption and Fluorescence spectroscopic study of Mito-ST and related Zn^{2+} titration

The stock solution of **Mito-ST** (1×10^{-4} M) was prepared by directly dissolving the sensor in DMSO. For the spectroscopic determination, the stock solution was diluted with HEPES buffer to the desired concentration (1×10^{-5} M). For Zn^{2+} titration, aliquots of 1 μL aqueous Zn^{2+} solution Zn $(NO_3)_2$ (1.2×10^{-3} M) were added to 3 mL diluted **Mito-ST** solution. The measurements were carried out in 1 min after the addition. All spectra were determined at 298 K.

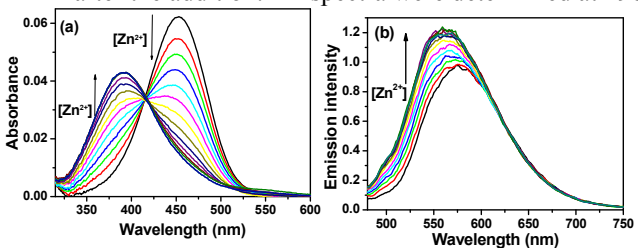


Figure S5. Absorption (**a**) and emission spectra (**b**, λ_{ex}, 466 nm) of 10 μM **Mito-ST** in HEPES buffer (50 mM HEPES, 100 mM KNO_3, pH 7.2) obtained upon titration with $Zn(NO_3)_2$ (1.2 mM) solution. The $[Zn^{2+}]_{total}$ values in the solution were increased from 0 to 25.0 μM along the direction of arrows.

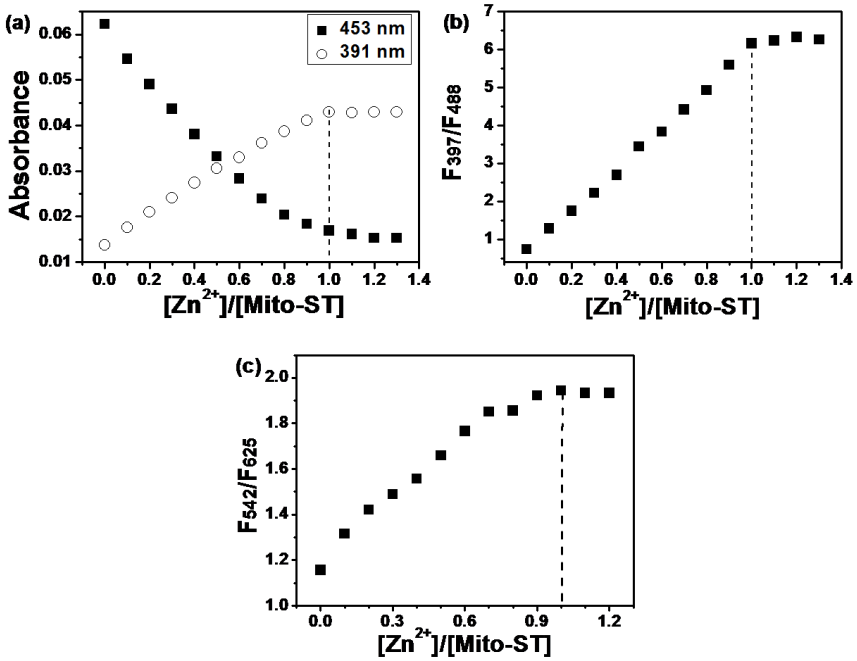


Figure S6. Titration profiles of 10 μM **Mito-ST** in HEPES buffer (50 mM HEPES, 100 mM KNO_3, pH 7.2) obtained upon titration with $Zn(NO_3)_2$ (1.2 mM) solution. (a) Titration profiles according to the absorbance at 391 and 453 nm; (b) titration profile according to the ratio of emission at 577 nm upon excitation at 397 to that upon excitation at 488 nm; (c) titration profile according to the ratio of emission at 542 to that at 625 nm upon excitation at 466 nm.

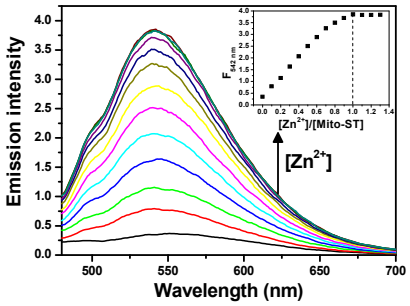


Figure S7. Emission spectra of 10 μM **Mito-ST** in HEPES buffer (50 mM HEPES, 100 mM M KNO_3, pH = 7.2, DMSO/H_2O, v/v 1:9) titrated by $Zn(NO_3)_2$ (1.2 mM) solution. The $[Zn^{2+}]_{total}$ values in the solution are 0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 22.5 and 25.0 μM (from bottom to top). Inset is the titration profile according to the fluorescence intensity at 542 nm ($F_{542\,nm}$), λ_{ex} = 397 nm.

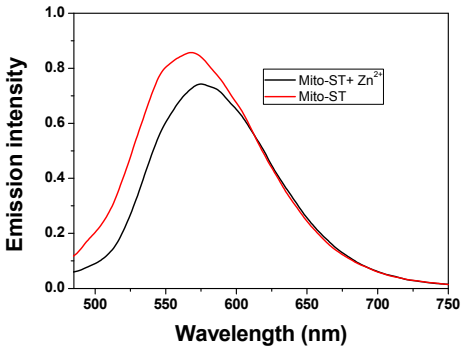


Figure S8. Emission spectra of 10 μM **Mito-ST** in HEPES buffer (50 mM HEPES, 100 mM M KNO_3, pH = 7.2, DMSO/H_2O, v/v 1:9) titrated by $Zn(NO_3)_2$ (1.2 mM) solution. λ_{ex} = 488 nm.

S5. Dissociation constant determination for Zn^{2+} complexes of Mito-ST in HEPES buffer

A series of buffered Zn^{2+} solutions were prepared for the determination of the dissociation constant. Thus, various amounts of $ZnNO_3$ (0 ~ 20 mM) were added to the solutions of (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) (HEPES 50 mM, pH 7.20, 0.1 M KNO_3) containing 10 mM of EGTA (ethylenebis(oxyethylenenitrilo) tetraacetic acid). The concentration of free Zn^{2+} was calculated with $[EGTA]_{total}$, $[Zn^{2+}]_{total}$, and $K'_{Zn\text{-}EGTA} = 3.80 \times 10^8\ M^{-1}$.

For the determination of dissociation constant, the above mentioned buffered Zn^{2+} solutions (3 mL) were added with 30 µL of free sensor solution (1 mM, DMSO as solvent). The final concentration of total sensor in the mixture is around 10 µM, and the emission spectra of the mixed solutions were determined respectively after complete mixing.

The apparent dissociation constants K_d with Zn^{2+} were determined by fitting the data to the following equation (Software: 1stOpt, calculate method: Levenberg-Marquardt):

$$F = F_0 + (F_{max} - F_0)[Zn^{2+}]_{free} = (K_d + [Zn^{2+}]_{free}) \qquad \text{(Eq. 1)}$$

where F is the observed fluorescence intensity, F_0 is the fluorescence intensity without Zn^{2+}, F_{max} is the maximum fluorescence intensity, and $[Zn^{2+}]_{free}$ is the concentration of free Zn^{2+}.

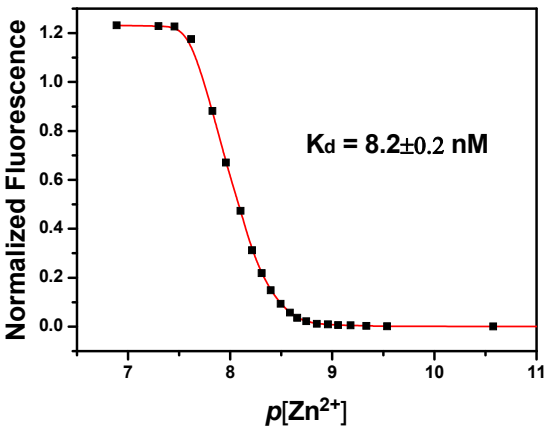


Figure S9. Normalized fluorescence intensity at 542 nm of **Mito-ST** (10 µM) as a function of free Zn^{2+} concentration in 50 mM HEPES buffer (pH 7.2, 0.1 M KNO_3) containing 10 mM EGTA and 0 - 20 mM $[Zn^{2+}]_{total}$.

S6. Zinc titration of Mito-ST determined by ^{1}H NMR spectroscopy

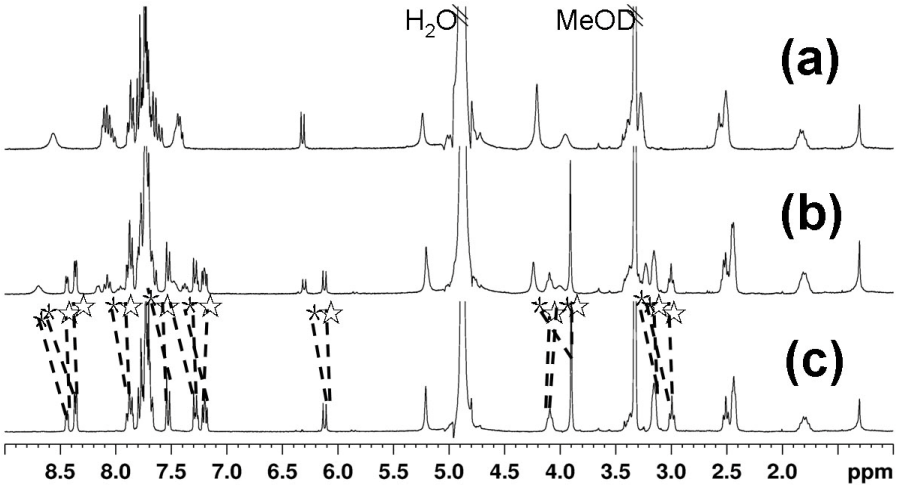


Figure S10. ^{1}H NMR spectra of **Mito-ST** (initial $c = 2.7 \times 10^{-2}$ M) in CD_3OD upon Zn^{2+} titration ($c = 9.0 \times 10^{-2}$ M in CD_3OD). (a) Spectrum obtained when the ratio of $[Zn^{2+}]_{total}$/[**Mito-ST**] is 1:1. (b) Spectrum obtained when the ratio of $[Zn^{2+}]_{total}$/[**Mito-ST**] equals 0.5:1. (c) Spectrum of free sensor. The signals marked with ☆ and * are for the protons from free sensor and zinc-bound sensor, respectively.

S7. Determination of quantum yield

Fluorescence quantum yield of Zn^{2+}/**Mito-ST** complex were determined in aqueous solutions (100 mM KNO_3, 50 mM HEPES, pH = 7.2) by using 4-methylamino-7-nitro-2,1,3-benzoxadiazole (Φ_f = 0.38, λ_{ex} = 458 nm, acetonitrile) as reference. The quantum yields were calculated using Eq.2:

$$\Phi_u = [(A_s F_u n^2)/ (A_u F_s n_0^2)]\Phi_s \quad \text{(Eq.2)}$$

Where A_s and A_u are the absorbance of the reference and sample solution at the reference excitation wavelength, F_s and F_u are the corresponding integrated fluorescence intensities, and n and n_0 are the refractive indexes of the solvents of the sample and the reference, respectively. All the emission spectra were obtained when excited at 458 nm. Absorbance of samples and reference at their respective excitation wavelengths were controlled to be lower than 0.05.

S8. Fluorescence of Mito-ST at different pH in DMSO–H_2O

Stock solutions of **Mito-ST** in DMSO (1×10^{-4} M) were diluted to a final concentration of 1×10^{-5} M with water. These solutions were adjusted to the desired pH by NaOH and HNO_3 solutions. The experiments were carried out at 298 K.

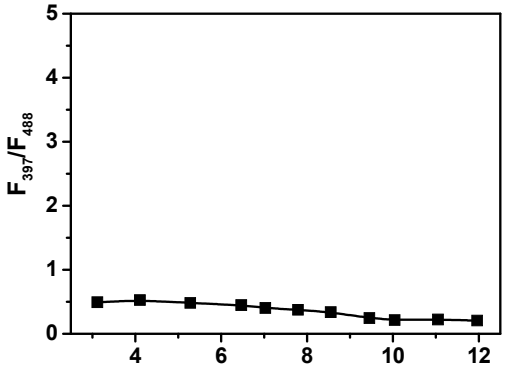


Figure S11. Emission ratio at 577 nm of 10 μM **Mito-ST** in aqueous medium upon irradiation at 397 and 488 nm (F_{397}/F_{488}).

S9. Confocal Imaging

HeLa, COC2 and MCF-7 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 mg/ml) and 5% CO_2 at 37 °C. After removing the incubation media and rinsing with 1× PBS for three times, the cells were stained with **Mito-ST** solution (20 μM, in 1 × PBS containing 0.5% DMSO) for 30 min at 25 °C. Then the cells were washed three times with PBS and imaged with Zeiss LSM-710 microscope equipped with a 60× oil-immersion objective.

Co-localization experiments were carried out by co-staining the cells with MitoTracker Red CMXRos (Invitrogen) and **Mito-ST**. The cells were incubated in 50 nM MitoTracker Red CMXRos solution at 25 °C for 20 min, then the cells were washed with 1× PBS for 2 times. After rinse with 1×PBS for 2 times, the cells were incubated with **Mito-ST** solution (20 μM) for 30 min at 25 °C. Finally, the cells were washed with 1×PBS for 2 times before imaging. The MitoTracker Red CMXRos marked images were obtained upon irradiation at 543 nm with a band path from 550 to 650 nm, while the **Mito-ST** stained images were obtained upon irradiation at 488 nm with a band path from 500-600 nm.

The dual exciation ratiometric imaging for intracelluar Zn^{2+} via **Mito-ST** staining (20 μM, 30 min, 25 °C) has been carried out in HeLa, MCF-7 and CoC2 cells. For the imaging of cells with the introduced exogenous Zn^{2+}, the exogenous Zn^{2+} was introduced by incubating the cells with 5 μM $ZnSO_4$/2-mercaptopyridine-*N*-oxide (1:1) solution (prepared by diluting 5 mM $ZnSO_4$/2-mercaptopyridine-*N*-oxide stock solution with 1 × PBS). Then, the cells were dyed with **Mito-ST** solution in a similar procedure described above and imaged. After the imaging, the cells of exogenous Zn^{2+} were treated with 50 μM TPEN solution (prepared by diluting the TPEN stock solution with 1 × PBS) for 10 min to scavenge Zn^{2+}. Then the cells were rinsed with 1 × PBS and imaged again. The excitation ratiometric imaging was carried out upon excitation at 405 and 488 nm, respectively, and images were collected with a band path from 500 to 600 nm.

The monitoring of mitochondrial Zn^{2+} release upon different stimulation was also carried out in MCF-7 cells with a dual excitation ratiometric imaging mode. Therefore, MCF-7 cells were firstly dyed with **Mito-ST** with the same procedure described above. Then the cells were incubated with SNOC (10 mM, 1h) or H_2O_2 (5 mM, 10 mM, and 50 mM, 20 min) at 25 ºC, and the Zn^{2+} release was tracked during the process by imaging with confocal microscope Zeiss LSM710. After that, the cells were treated with 50 μM TPEN solution to scavenge the intracellular Zn^{2+}. This ratiometric imaging was carried out using the same imaging mode aforementioned with the same imaging parameters.

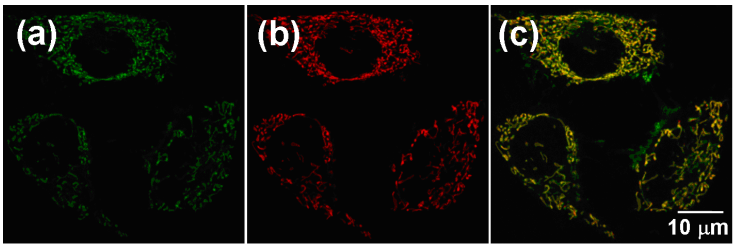


Figure S12. Confocal fluorescence images of HeLa cells co-stained by **Mito-ST** (20 μM, 30 min) and Mitotracker Red CMXRos (50 nM, 20 min) at 25 °C: (**a**) image based on the fluorescence of **Mito-ST** recorded at 500-600 nm, λ_{ex}, 488 nm; (**b**) image based on the fluorescence of Mitotracker recorded at 550-650 nm, λ_{ex}, 543 nm; (**c**) overlay of (**a**) and (**b**).

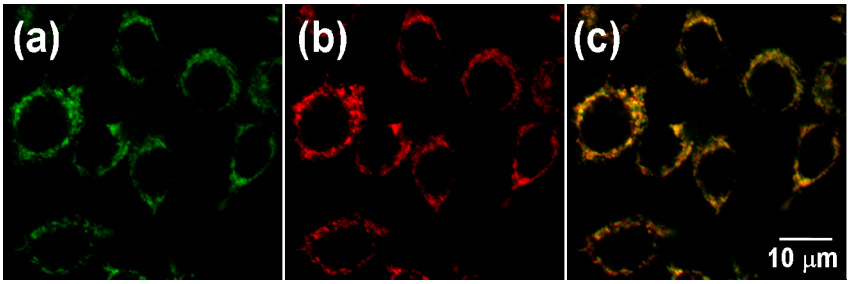


Figure S13. Confocal fluorescence images of COC2 cells costained by **Mito-ST** (20 μM, 30 min) and Mitotracker Red CMXRos (50 nM, 20 min) at 25°C. (a) Image for **Mito-ST** collected at 500-600 nm upon irradiation at 488 nm; (b) image for Mitotracker collected at 550-650 nm upon irradiation at 543 nm; (c) overlay of (a) and (b).

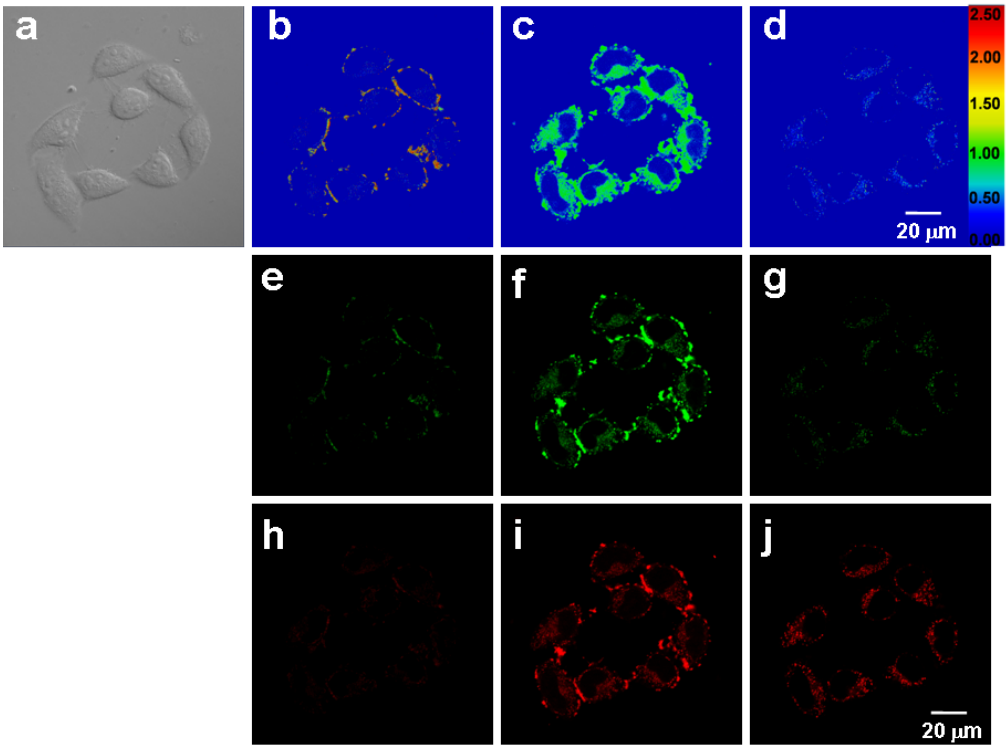


Figure S14. Confocal fluorescence images of HeLa cells stained with **Mito-ST** (20 μM) at 25 °C for 30 min. (a) Bright-field image of the stained cells; (e) and (h) fluorescence images of the stained cells; (f) and (i) fluorescence images of stained cells treated by $ZnSO_4$/pyrithione (5 μM, 1:1, 5 min) at 25 °C followed by washing with **Mito-ST** solution; (g) and (j) fluorescence images of cells in (f) and (i) treated further by TPEN solution (50 μM, 10 min); (e), (f), and (g) fluorescence images obtained according to the emission at 500–600 nm upon irradiation at 405 nm); (h), (i), and (j) fluorescence images obtained according to the emission collected at 500–600 nm upon irradiation at 488 nm; (b), (c), and (d) ratiometric images generated from (e) and (h), (f) and (i), and (g) and (j), respectively.

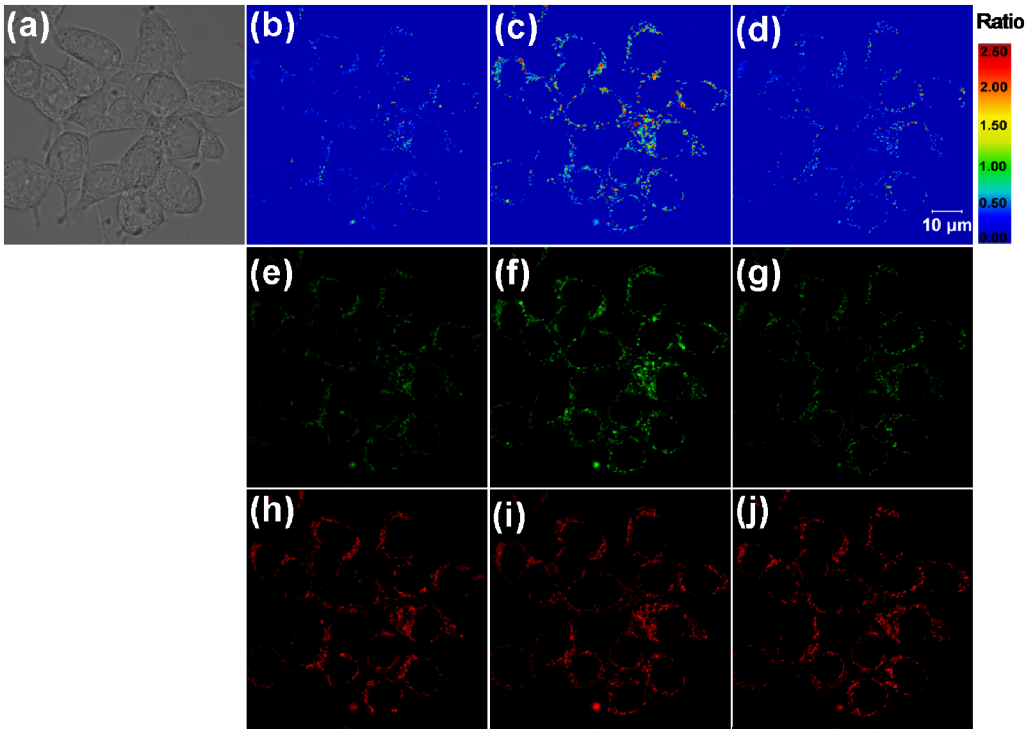


Figure S15. Confocal fluorescence images of COC2 cells stained with **Mito-ST** (20 μM) at 25 °C for 30 min. (a) Bright-field image of the stained cells; (e) and (h) fluorescence images of the stained cells; (f) and (i) fluorescence images of stained cells treated by $ZnSO_4$/pyrithione (5 μM, 1:1, 5 min) at 25 °C followed by washing with **Mito-ST** solution; (g) and (j) fluorescence images of cells in (f) and (i) treated further by TPEN solution (50 μM, 10 min); (e), (f), and (g) fluorescence images obtained according to the emission at 500–600 nm upon irradiation at 405 nm); (h), (i), and (j) fluorescence images obtained according to the emission collected at 500–600 nm upon irradiation at 488 nm; (b), (c), and (d) ratiometric images generated from (e) and (h), (f) and (i), and (g) and (j), respectively.

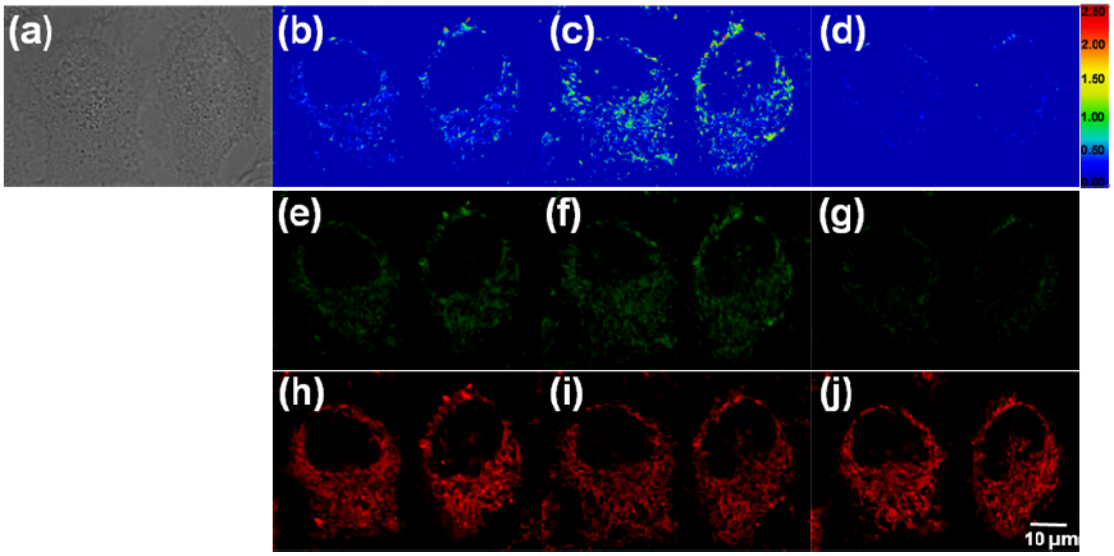


Figure S16. Confocal fluorescence images of MCF-7 cells stained with **Mito-ST** (20 μM) at 25 °C for 30 min. (a) Bright-field image of the stained cells; (e) and (h) fluorescence images of the stained cells; (f) and (i) fluorescence images of stained cells treated by $ZnSO_4$/pyrithione (5 μM, 1:1, 5 min) at 25 °C followed by washing with **Mito-ST** solution; (g) and (j) fluorescence images of cells in (f) and (i) treated further by TPEN solution (50 μM, 10 min); (e), (f), and (g) fluorescence images obtained according to the emission at 500–600 nm upon irradiation at 405 nm; (h), (i), and (j) fluorescence images obtained according to the emission collected at 500–600 nm upon irradiation at 488 nm; (b), (c), and (d) ratiometric images generated from (e) and (h), (f) and (i), and (g) and (j), respectively.

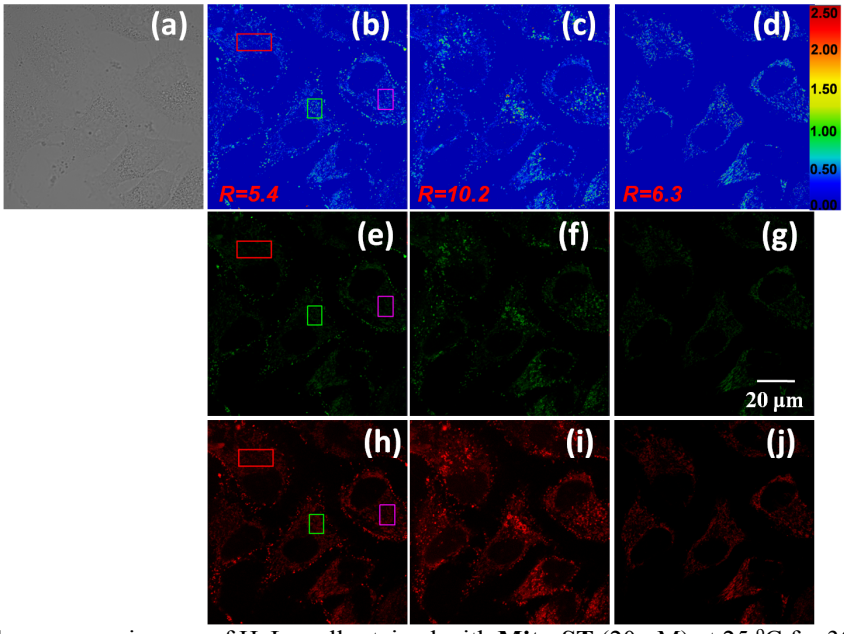


Figure S17. Confocal fluorescence images of HeLa cells stained with **Mito-ST** (20 μM) at 25 °C for 30 min upon irradiation at 488 nm. (a) Bright-field image of the stained cells; (e) and (h) fluorescence images of the stained cells; (f) and (i) fluorescence images of stained cells treated by $ZnSO_4$/pyrithione (5 μM, 1:1, 5 min) at 25 °C followed by washing with **Mito-ST** solution; (g) and (j) fluorescence images of cells in (f) and (i) treated further by TPEN solution (50 μM, 10 min); (e), (f), and (g) fluorescence images obtained with a band path of 510-570 nm; (h), (i), and (j) fluorescence images obtained with a band path of 600–650 nm upon irradiation at 488 nm; (b), (c), and (d) ratiometric images generated from (e) and (h), (f) and (i), and (g) and (j), respectively, and the R values in red shown in (b), (c) and (d) are the average emission ratio values of regions of interest.

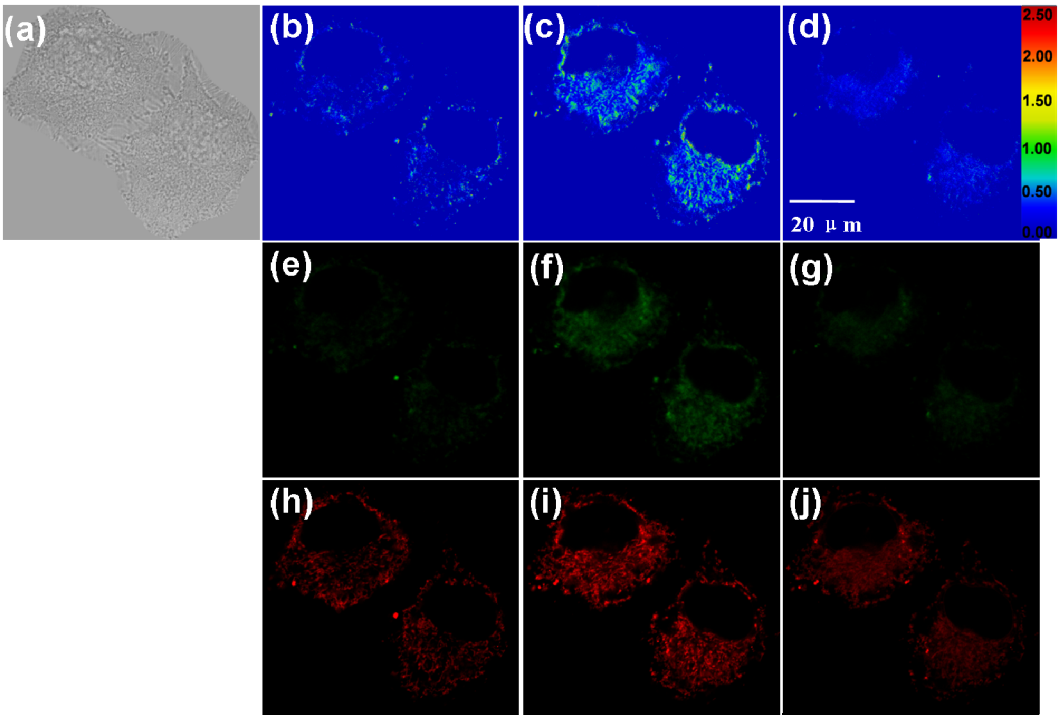


Figure S18. Confocal fluorescence images of **Mito-ST-**stained MCF-7 cells upon stimulation by H_2O_2. (a) Bright-field image of the stained cells (20 μM **Mito-ST**, 25 ^{o}C for 30 min); (e) and (h) fluorescence images of the stained cells; (f) and (i) fluorescence images of stained cells treated 10 mM H_2O_2 solution at 25 ^{o}C for 20 min; (g) and (j) fluorescence images of cells in (f) and (i) treated further by TPEN solution (50 μM, 10 min); (e), (f), and (g) fluorescence images obtained according to the emission at 500–600 nm upon irradiation at 405 nm; (h), (i), and (j) fluorescence images obtained according to the emission collected at 500–600 nm upon irradiation at 488 nm. (b), (c), and (d) ratiometric images generated from (e) and (h), (f) and (i), and (g) and (j), respectively.

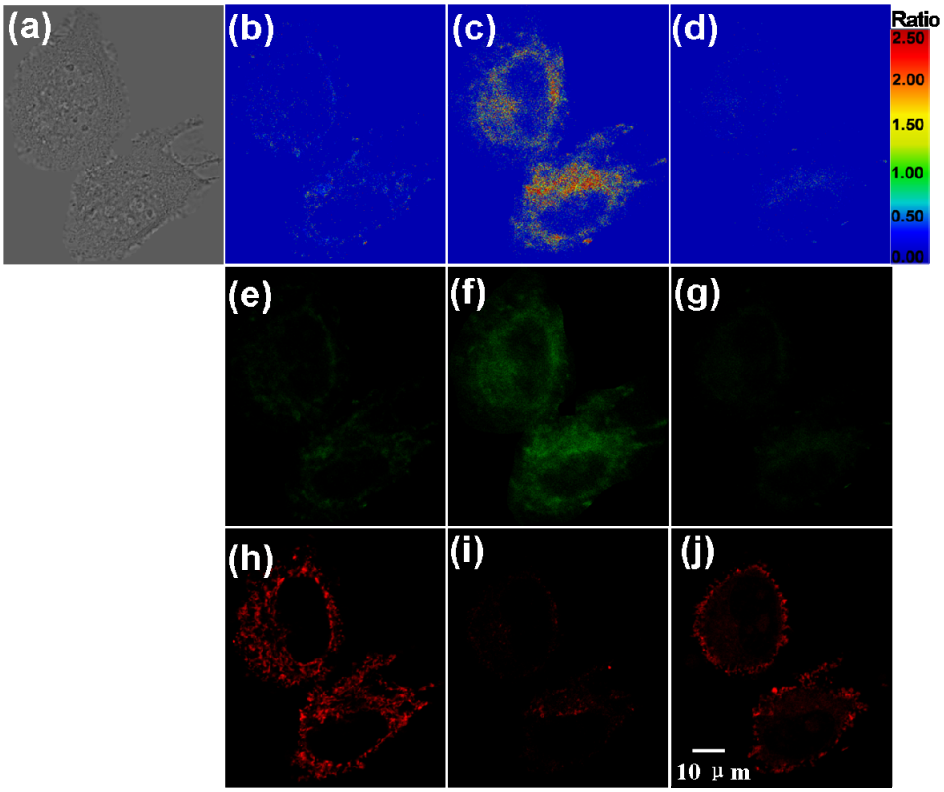


Figure S19. Confocal fluorescence images of **Mito-ST**-stained MCF-7 cells upon stimulation by SNOC. (a) Bright-field image of the stained cells (**Mito-ST** 20 μM, 25 ^{o}C, 30 min); (e) and (h) fluorescence images of the stained cells; (f) and (i) fluorescence images of stained cells treated by 10 mM SNOC solution at 25 ^{o}C for 1 h; (g) and (j) fluorescence images of cells in (f) and (i) treated further by TPEN solution (50 μM, 10 min); (e), (f), and (g) fluorescence images obtained according to the emission at 500–600 nm upon irradiation at 405 nm; (h), (i), and (j) fluorescence images obtained according to the emission collected at 500–600 nm upon irradiation at 488 nm; (b), (c), and (d) ratiometric images generated from (e) and (h), (f) and (i), and (g) and (j), respectively.