Supporting Information

Rational design of a ratiometric two-photon fluorescent probe for real-time visualization of apoptosis

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List of contents

1. Theoretical calculation
2. Synthesis of Mito-SCHO and Mito-DCHO
3. Experimental Section
4. Time-dependent fluorescent response of Mito-SCHO to Cys
5. Time-dependent UV-vis spectra of Mito-SCHO and Mito-DCHO to Cys/Hcy
6. Chemical stability and competitive experiment
7. Fluorescence and UV-vis absorption spectra of Mito-DCHO to Cys concentrations
8. Determination of detection limit
9. Determination of pH effect
10. ¹H-NMR titration
11. ESI-MS spectra of Mito-DCHO, Mito-DCHO-Cys, Mito-SCHO and Mito-SCHO-Cys/Hcy
12. Two-photon absorption properties
13. Cytotoxicity Assays
14. TPM imaging of Mito-DCHO’s mitochondria-targeted capability
15. Dual-color imaging of Mito-DCHO to exogenous and endogenous Cys in cells
16. Dual-color imaging of mitochondrial Cys under oxidative stress induced by H₂O₂
17. Dual-color imaging of mitochondrial oxidative stress levels under LPS-induced apoptosis
18. Ratiometric TPM images in zebrafish during LPS-induced apoptosis
19. NMR Spectra and mass original spectra
1. Theoretical calculation

![Diagram](image)

**Fig. S1** (a) Reaction of CA1 with Cys. (b) Calculated HOMO and LUMO of CA1 and CA1 + Cys.

To better understand the behavior of three probes for sensing Cys, DFT calculations using the M06-D3 method and the def2-SVP basis set with the Gaussian 09 program package have been carried out.\(^1,2\)

As shown in Scheme 1A and Fig. S1, electrons were mainly centralized at the carbazole framework as the donor (D) at the ground state and transferred to the pyridine moiety (A2, as the major part) and aldehyde group (A1, as the minor part) as the acceptors after excitation, indicating a “A2-π-D-π-A1”-typed two-dimensional ICT process for CA1. After CA1 reacting with Cys to obtain product CA1+Cys, the electrons were still localized over the carbazole at the ground state, which transferred to the pyridine moiety (A2) only at the excitation state. The result indicated the main ICT system between carbazole and pyridine was unchanged before and after CA1 reacting with Cys. At the same time, the energy gap between the HOMO and LUMO of CA1 was calculated to be 4.49 eV and that of its product was 4.43 eV. There are
very small differences between two energy gap values, indicating a weak ICT efficiency changes in this reaction. Therefore, we envisioned that the fail of the ratiometric response of CA1 to Cys was endowed to the remaining of strong ICT efficiency between carbazole and pyidine moieties.

On the other hand, for our probe Mito-DCHO, the DFT calculations showed the different results. As shown in Scheme 1A and Fig. S2, electrons were mainly centralized at the carbazole framework as the donor (D) at the ground state and transferred to the two aldehyde groups (A1) as the acceptors after excitation, indicating a “A1-π-D-π-A1”-typed two-dimensional ICT process for Mito-DCHO. After the reaction of probe with Cys to obtain product Mito-DCHO-Cys, electrons distribution of Mito-DCHO-Cys changed to a large extent. The electrons were mainly localized over the carbazole and phenylacetylene group both at the ground and excitation state, which indicated its two-dimensional ICT system was off. Moreover, the energy gap between the HOMO and LUMO of Mito-DCHO was calculated to be 3.86 eV and that of Mito-DCHO-Cys was 4.24 eV. There are much bigger differences between two energy gap values, indicating a strong ICT efficiency changes happened. More importantly, the strong ICT efficiency changes endowed Mito-DCHO with ratiometric fluorescent response to Cys along with a large emission shift (119 nm). Mito-SCHO had the similar DFT calculation results. Its fluorescent signal for Cys was consistent with that of Mito-DCHO to Cys. However, Mito-SCHO with a one-dimensional ICT system cannot discriminate Cys over Hcy for their similar reaction kinetics.

As a result, the DFT calculation supported our rational design that a dialdehyde-functionalized carbazole derivative can serve as a Cys-specific ratiometric fluorescent probe employing a symmetric two-dimensional ICT system.
Fig. S2 (a) Calculated HOMO and LUMO of Mito-DCHO and Mito-DCHO-Cys. (b) Calculated HOMO and LUMO of Mito-SCHO and Mito-SCHO-Cys.
2. Synthesis of **Mito-SCHO** and **Mito-DCHO**

**Scheme S1** Synthesis route of **Mito-SCHO** and **Mito-DCHO**.

2.1 Synthesis of compound 1

A mixture of KOH (2.88 g, 51.4 mmol), KI (0.57 g, 3.4 mmol) and 1,4-dibromobutane (11.1 g, 51.4 mmol) was heated to 60 °C. After reacting 1 h, 3-iodocarbazole (10 g, 34.3 mmol) was added slowly, and the result mixture was refluxed until TLC showed no raw material existed (about 12 h). After cooling to room temperature, 200 mL H₂O was added, and the mixture was extracted with dichloromethane (100 mL×2). The organic phase was evaporated to give crude product. The crude material was purified by column chromatography (petroleum ether:dichloromethane = 10 : 1 as the eluent) to give 6.4 g (43.6%) of 1.
H NMR (400 MHz, CDCl₃, ppm): δ 8.39 (s, 1H), 8.03 (d, J = 7.8 Hz, 1H), 7.70 (d, J = 8.7 Hz, 1H), 7.49 (t, J = 7.6 Hz, 1H), 7.39 (m, J = 8.1 Hz, 1H), 7.24 (t, J = 10.9 Hz, 1H), 7.18 (d, J = 8.5 Hz, 1H), 4.32 (t, J = 6.8 Hz, 2H), 3.37 (t, J = 6.4 Hz, 2H), 2.04 (m, J = 14.5, 7.4 Hz, 2H), 1.89 (m, J = 13.2, 6.6 Hz, 2H).

C NMR (100 MHz, CDCl₃, ppm): δ 140.27, 139.44, 133.93, 129.30, 126.48, 125.45, 121.67, 120.62, 119.53, 110.60, 108.74, 81.41, 42.27, 32.97, 30.14, 27.55.

2.2 Synthesis of compound 2

A mixture of compound 1 (1 g, 2.3 mmol), 4-ethynylbenzaldehyde (0.46 g, 3.5 mmol), Pd(PPh₃)₂Cl₂ (6.6 mg, 0.009 mmol), CuI (3.6 mg, 0.018 mmol) and Et₃N (5 ml) was stirred at 30 °C for 12 h under the anhydrous and anaerobic conditions. The mixture was cooled to room temperature. Precipitates were filtered off and concentrated to give the crude product. The crude material was purified by column chromatography (petroleum ether:dichloromethane = 4 : 1 as the eluent) to give 600 mg (59.7%) of 2.

H NMR (400 MHz, CDCl₃, ppm): δ 9.93 (s, 1H), 8.24 (s, 1H), 8.02 (d, J = 7.8 Hz, 1H), 7.78 (d, J = 7.5 Hz, 2H), 7.59 (d, J = 16.5, 8.0 Hz, 3H), 7.42 (t, J = 7.7 Hz, 1H), 7.31 (m, J = 14.1, 8.4 Hz, 2H), 7.22-7.16 (t, 1H), 4.25 (m, J = 13.1, 6.5 Hz, 2H), 3.30 (t, J = 6.5 Hz, 1H), 3.07 (t, J = 6.7 Hz, 1H), 2.02-1.90 (m, 2H), 1.87-1.75 (m, 2H). C NMR (100 MHz, CDCl₃, ppm): δ 191.48, 140.71, 140.33, 135.01, 131.86, 130.37, 129.65, 129.55, 126.47, 124.49, 123.07, 122.48, 120.66, 119.80, 112.64, 108.93, 108.77, 95.38, 87.24, 42.16, 32.97, 27.62, 5.72.

2.3 Synthesis of Mito-SCHO

A mixture of 2 (1 g, 2.3 mmol) and acetonitrile (10 ml) was heated to 80 °C under
the condition of nitrogen. After 1 h, PPh$_3$ (3.7 g, 13.9 mmol) was added, the result mixture was refluxed until TLC showed no raw material existed (about 36 h). After being cooled to room temperature, the mixture was filtered and the filtrate was evaporated to give the crude product. The crude product was purified through column chromatography (dichloromethane:acetonitrile = 3 : 1 as the eluent) to give 0.96 g (60%) of **Mito-SCHO**. ESI-MS m/z: [**Mito-SCHO** - Br]$^+$ C$_{43}$H$_{35}$NOP$^+$ calcd, 612.2456; found, 612.2427.

$^1$H NMR (400 MHz, DMSO-$d_6$, ppm): δ 10.05 (s, 1H), 8.46 (s, 1H), 8.22 (d, $J$ = 7.9 Hz, 1H), 7.98 (d, $J$ = 7.3 Hz, 2H), 7.87 (t, $J$ = 6.5 Hz, 3H), 7.79 (d, $J$ = 7.5 Hz, 2H), 7.73-7.62 (m, 15H), 7.48 (t, $J$ = 7.7 Hz, 1H), 7.26 (t, $J$ = 7.7 Hz, 1H), 4.49 (t, $J$ = 6.1 Hz, 2H), 3.60 (t, $J$ = 13.6 Hz, 2H), 1.96 (m, $J$ = 13.6, 7.2 Hz, 2H), 1.61 (m, $J$ = 11.1 Hz, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$, ppm): δ 191.49, 140.71, 140.43, 135.02, 135.00, 133.55, 133.45, 131.84, 130.46, 130.34, 130.27, 129.68, 129.59, 126.51, 124.18, 122.78, 122.27, 120.39, 119.70, 118.08, 117.23, 112.45, 109.64, 109.56, 95.33, 87.28, 42.25, 29.34, 23.12, 20.13.

### 2.4 Synthesis of compound 3

A mixture of KOH (2.0 g, 35.8 mmol), KI (0.4 g, 2.39 mmol) and 1,4-dibromobutane (7.73 g, 35.8 mmol) was heated to 60 °C. After reacting 1 h, 3,6-diiodocarbazole (10 g, 23.9 mmol) was added slowly, the result mixture was refluxed until TLC showed no raw material existed (about 12 h). After being cooled to room temperature, 200 mL H$_2$O was added, the mixture was extracted with dichloromethane (100 mL×2). The organic phase was evaporated to give crude product. The crude material was purified by column chromatography (petroleum ether:dichloromethane = 10 : 1 as the eluent) to give 6.8 g (51.4%) of **3**.
1H NMR (400 MHz, CDCl3, ppm): δ 8.34 (d, J = 1.5 Hz, 2H), 7.74 (d, J = 1.6 Hz, 1H), 7.71 (d, J = 1.6 Hz, 1H), 7.19 (s, 1H), 7.17 (s, 1H), 4.29 (t, J = 7.0 Hz, 2H), 3.37 (t, J = 6.4 Hz, 2H), 2.06-1.98 (m, 2H), 1.86 (m, J = 13.1, 6.5 Hz, 2H). 13C NMR (100 MHz, CDCl3, ppm): δ 139.38, 134.69, 129.48, 124.08, 110.76, 81.93, 42.37, 32.82, 30.02, 27.44.

2.5 Synthesis of compound 4

A mixture of compound 3 (1 g, 1.8 mmol), 4-ethynylbenzaldehyde (0.7 g, 5.4 mmol), Pd(PPh3)2Cl2 (5.1 mg, 0.007 mmol), CuI (2.7 mg, 0.014 mmol) and Et3N (5 ml) was stirred at 30 °C for 12 h under the anhydrous and anaerobic conditions. the mixture was cooled to room temperature. Precipitates were filtered off and concentrated to give the crude product. The crude material was purified by column chromatography (petroleum ether:dichloromethane = 4 : 1 as the eluent) to give 0.65 g (64.5%) of 4.

1H NMR (400 MHz, CDCl3, ppm): δ 10.04 (s, 2H), 8.33 (s, 2H), 7.89 (t, J = 7.4 Hz, 4H), 7.71 (m, J = 12.4, 5.8 Hz, 6H), 7.42 (d, J = 8.5 Hz, 2H), 4.38 (m, J = 12.4, 6.6 Hz, 2H), 3.42 (t, J = 6.3 Hz, 1H), 3.19 (t, J = 6.6 Hz, 1H), 2.15-2.01 (m, 2H), 1.92 (m, J = 13.5, 6.7 Hz, 2H). 13C NMR (100 MHz, CDCl3, ppm): δ 191.47, 140.67, 135.13, 133.12, 131.92, 130.18, 130.14, 129.67, 129.59, 124.63, 122.62, 113.56, 109.14, 94.83, 87.58, 32.84, 30.74, 27.59, 5.54.

2.6 Synthesis of Mito-DCHO

A mixture of 4 (1 g, 1.8 mmol) and acetonitrile (10 mL) was heated to 80 °C under the condition of nitrogen. After 1 h, PPh3 (2.8 g, 10.8 mmol) was added, the result mixture was refluxed until TLC showed no raw material existed (about 36 h). After
being cooled to room temperature, the mixture was filtered and the filtrate was evaporated to give crude product. The crude product was purified through column chromatography (dichloromethane:acetonitrile = 3:1 as the eluent) to give 0.83 g (56%) of Mito-DCHO.

1H NMR (400 MHz, DMSO-d6, ppm): δ 10.05 (s, 2H), 8.53 (s, 2H), 7.98 (d, J = 8.0 Hz, 4H), 7.87 (m, J = 8.6, 4.6 Hz, 3H), 7.79 (d, J = 8.0 Hz, 4H), 7.76-7.68 (m, 16H), 4.51 (t, J = 6.9 Hz, 2H), 3.62 (t, J = 15.1 Hz, 2H), 2.00-1.93 (m, 2H), 1.62 (m, J = 14.1, 6.2 Hz, 2H). 13C NMR (100 MHz, CDCl3, ppm): δ 191.43, 140.73, 135.13, 135.08, 135.05, 133.60, 133.50, 132.15, 132.05, 131.88, 130.50, 130.37, 130.20, 130.08, 129.67, 128.57, 128.45, 124.24, 122.31, 118.10, 117.24, 113.34, 110.05, 94.88, 87.59, 42.55, 29.19, 22.66, 20.15. ESI-MS m/z: [Mito-DCHO - Br]⁺ C52H33NO2P⁺ calcd, 740.2718; found, 740.2702.

3. Experimental Section

3.1. General procedures

All reagents and solvents were commercially purchased. 1H-NMR spectra were recorded on Bruker-400 MHz spectrometers and 13C-NMR spectra were recorded on 100 MHz spectrometers. Fluorescence spectra were obtained using a HITACHIF-2500 spectrometer. UV-vis absorption spectra were recorded on a Tech-comp UV 1000 spectrophotometer. MS spectra were conducted by ESI mass spectrometer.

The test solution of Mito-SCHO and Mito-DCHO (10 μM) in buffer solution (pH=7.4, DMSO/PBS, 1:1, v/v) was prepared. The solutions of various testing species were prepared for Cys, Hey, GSH, Pro, Met, Val, Ala, Asp, Trp, Tyr, Ile, His, Thr, Ser, Hyp, Leu, Phe, SCN⁻, S²⁻, SO₄²⁻, S₂O₃²⁻, SO₃²⁻, CH₃COO⁻,
Cl\(^{−}\), CO\(_3^{2−}\), NO\(_3^{−}\), NO\(_2^{−}\), Cu\(^{2+}\), Na\(^{+}\), Mg\(^{2+}\) and Fe\(^{3+}\). Various ROS including H\(_2\)O\(_2\), ClO\(^{−}\), O\(_2^{−}\), TBHP, HO\(^{·}\), t-BuO\(^{·}\) and CH\(_3\)COOOH were prepared according to the procedure reported previously.\(^3\) The resulting solution was shaken well and incubated for 20 min at room temperature before recording the spectra.

3.2. Measurement of two-photon absorption cross-section (δ)

Two-photon excitation fluorescence (TPEF) spectra were measured using femtosecond laser pulse and Ti: sapphire system (680~1080 nm, 80 MHz, 140 fs, Chameleon II) as the light source. All measurements were carried out in air at room temperature. Two-photon absorption cross-sections were measured using two-photon-induced fluorescence measurement technique. The two-photon absorption cross-sections (δ) were determined in DMSO with Mito-DCHO/Mito-DCHO (0.1 mM) with the addition of Cys (0.6 mM) by using optically matching solutions of fluorescein as a standard, according to the following equation:

\[
\delta = \delta_{\text{ref}} \frac{\Phi_{\text{ref}}}{\Phi} \frac{c_{\text{ref}}}{c} \frac{n_{\text{ref}}}{n} \frac{F}{F_{\text{ref}}}
\]

Here, the subscripts ref stands for the reference molecule. δ is the TPA cross-section value, c is the concentration of solution, n is the refractive index of the solution, F is the TPEF integral intensities of the solution emitted at the exciting wavelength, and Φ is the fluorescence quantum yield. The Φ\(_{\text{ref}}\) value of reference (Φ\(_{\text{fluorescein}} = 0.97\)) is taken from the literature.\(^4\) The δ\(_{\text{ref}}\) value of reference was taken from the literature,\(^5\) and the relative error of δ value in the experiment is about ±15%.
3.3. Cytotoxicity assay

MTT (5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide) assay was performed as previously reported to test the cytotoxic effect of the probe in cells.\textsuperscript{6} HeLa cells were passed and plated to ca. 70% confluence in 96-well Plates 24 h before treatment. Prior to Mito-DCHO treatment, DMEM (Dulbecco’s Modified Eagle Medium) with 10% FCS (Fetal Calf Serum) was removed and replaced with fresh DMEM, and aliquots of Mito-DCHO stock solutions (1 mM DMSO) were added to obtain final concentrations of 10, 20 and 30mM respectively. The treated cells were incubated for 24 h at 37°C under 5%CO\textsubscript{2}. Subsequently, cells were treated with 5 mg/mL MTT (40 mL/well) and incubated for an additional 4 h (37 °C, 5% CO\textsubscript{2}). Then the cells were dissolved in DMSO (150 mL/well), and the absorbance at 570 nm was recorded. The cell viability (%) was calculated according to the following equation:

\[
\text{Cell viability} \% = \frac{\text{OD}_{570}(\text{sample})}{\text{OD}_{570}(\text{control})} \times 100 \%
\]

where \text{OD}_{570}(\text{sample}) represents the optical density of the wells treated with various concentration of Mito-DCHO and \text{OD}_{570}(\text{control}) represents that of the wells treated with DMEM containing 10% FCS. The percent of cell survival values is relative to untreated control cells.

3.4. Cell culture and two-photon fluorescence microscopy imaging

For two-photon imaging, HeLa cells were cultured in DMEM supplemented with 10% FCS, penicillin (100 µg/mL), and streptomycin (100 µg/mL) at 37°C in a humidified atmosphere with 5% CO\textsubscript{2} and 95% air. Cytotoxicity assays show that Mito-DCHO is safe enough for two-photon bio-imaging at low concentrations. The cells were first washed with PBS, incubated with probe,
N-ethylmaleimide (NEM), thiol and H₂O₂ respectively in the incubator at 37 °C and then rinsed for three times with PBS. Cells imaging was carried out on a confocal microscope (Zeiss LSM 510 Meta NLO). Two-photon fluorescence microscopy images of labeled cells were obtained by exciting the probe with a mode-locked titanium–sapphire laser source at 720 nm.

3.5. Dual-color imaging of mitochondrial oxidative stress levels under LPS-induced apoptosis

TPM images of mitochondrial oxidative stress levels under LPS-induced apoptosis utilizing probe **Mito-DCHO**. Cells were incubated with **Mito-DCHO** (10 μM) for 30 min, and imaged. Cells were mediated by LPS (5 μg/mL) for 0.5, 1.0, 1.5, 2.0 and 2.5 h, respectively, subsequently incubated with **Mito-DCHO** (10 μM) for 30 min and imaged. Cells imaging was carried out on a confocal microscope (Zeiss LSM 510 Meta NLO). Two-photon fluorescence microscopy images of labeled cells were obtained by exciting the probe with a mode-locked titanium–sapphire laser source at 720 nm.

3.6. Ratiometric TPM images in zebrafish during LPS-induced apoptosis

All procedures involving animals were approved by and conformed to the guidelines of the Anhui University Animal Care Committee, School of life science. We have taken great efforts to reduce the number of animal used in these studies and also taken effort to reduce animal suffering from pain and discomfort.

Ratiometric TPM images in zebrafish during LPS-induced apoptosis. Zebrafishes were mediated by LPS (100 μg/mL) for 0, 0.5, 1, 1.5 and 2 h, respectively, subsequently incubated with **Mito-DCHO** (20 μM) for 0.5 h. All
the fishes were terminally anaesthetized using MS222, and images were carried out on a confocal microscope (Zeiss LSM 710 Meta NLO). Two-photon fluorescence microscopy images of zebrafishes were obtained by exciting Mito-DCHO with a mode-locked titanium–sapphire laser source at 720 nm.

References

4. Time-dependent fluorescent response of **Mito-SCHO** to Cys

*Fig. S3* Time-dependent fluorescence spectra response of **Mito-SCHO** (10 μM, λ<sub>ex</sub> = 345 nm) with (a) Cys (60 μM) and (b) Hcy (60 μM) in PBS/DMSO (5:5, v/v, pH=7.4). Inset: Relationships between time and the emission ratio (I<sub>390nm</sub>/I<sub>521nm</sub>) changes of **Mito-SCHO** towards Cys (a) and Hcy (b).

5. Time-dependent UV-vis spectra of **Mito-SCHO** and **Mito-DCHO** to Cys/Hcy

*Fig. S4* Time-dependent UV-vis absorption response of (a) **Mito-SCHO** (10 μM) with Cys (60 μM) and (b) **Mito-DCHO** (10 μM) with Cys (60 μM).
μM), (b) **Mito-SCHO** (10 μM) with Hcy (60 μM), (c) **Mito-DCHO** (10 μM) with Cys (60 μM), (d) **Mito-DCHO** (10 μM) with Hcy (60 μM) in PBS/DMSO (5:5, v/v, pH=7.4). Inset: Relationships between time and the absorbance ratio (A320nm/A373nm) changes of (a) **Mito-SCHO** with Cys, (b) **Mito-SCHO** with Hcy; Relationships between time and the absorbance ratio (A313nm/A373nm) changes of (c) **Mito-DCHO** with Cys, (d) **Mito-DCHO** with Hcy.

6. Chemical stability and competitive experiment

![Fluorescence spectra of Mito-DCHO](image)

**Fig. S5** Fluorescence spectra of **Mito-DCHO** (10 μM) upon addition of various ROS (60 μM, including H2O2, ClO-, O2•-, TBHP, HO•, t-BuO• and CH3COOOH) in PBS/DMSO buffer solution (5:5, v/v, pH=7.4, λex = 345 nm).

![Emission ratios](image)

**Fig. S6** Emission ratios (I402nm/I521nm) of **Mito-DCHO** (10 μM) upon addition of analytes (60 μM)
in PBS/DMSO buffer solution (5:5, v/v, pH=7.4, λex = 345 nm). Black and red bars represent the fluorescence ratio of probe in the presence of various analytes before and after addition of Cys, respectively. Each spectrum was acquired 20 min after addition of various analytes. 1, Cys; 2, Hcy; 3, GSH; 4, Pro; 5, Met; 6, Val; 7, Ala; 8, Asp; 9, Trp; 10, Tyr; 11, Ile; 12, His; 13, Thr; 14, Ser; 15, Hyp; 16, Leu; 17, Phe; 18, SCN⁻; 19, S²⁻; 20, SO4²⁻; 21, S₂O₃²⁻; 22, SO₃²⁻; 23, CH₃COO⁻; 24, Cl⁻; 25, CO₃²⁻; 26, NO₃⁻; 27, NO₂⁻; 28, Cu²⁺; 29, Na⁺; 30, Mg²⁺; 31, Fe³⁺.

7. Fluorescence and UV-vis absorption spectra of **Mito-DCHO** to Cys concentrations

![Fluorescence spectra](image1.png)

**Fig. S7** Fluorescence spectra (a) and UV-vis absorption spectra (b) of **Mito-DCHO** (10 μM) in the presence of increasing amount of Cys (0~60 μM) in PBS/DMSO (5:5, v/v, pH=7.4, λex = 345 nm).

Inset: Relationship between the emission ratio (I₄02nm/I₅21nm) changes of **Mito-DCHO** (a) with Cys, and the relationship between the absorbance ratio (A₃13nm/A₃73nm) changes of **Mito-DCHO** (b) with Cys concentrations. Spectra were recorded for 20 min after Cys addition.
8. Determination of detection limit

![Graph](image)

**Fig. S8** Emission ratio (I\textsubscript{402nm}/I\textsubscript{521nm}) changes of Mito-DCHO (10 μM) upon addition of Cys (0–5 μM) in PBS/DMSO (5:5, v/v, pH=7.4, λ\textsubscript{ex} = 345 nm).

9. Determination of pH effect

![Graph](image)

**Fig. S9** Emission ratio (I\textsubscript{402nm}/I\textsubscript{521nm}) of Mito-DCHO (10 μM) at various pH values in the absence (black) and presence (red) of Cys (60 μM) in PBS/DMSO buffer (5/5, v/v, pH=7.4, λ\textsubscript{ex} = 345 nm).

Each spectrum was recorded after 20 min.
10. $^1$H-NMR titration

![H-NMR spectra of (a) Mito-DCHO and (b) Mito-DCHO-Cys in DMSO-d$_6$.](image)

Fig. S10 $^1$H NMR spectra of (a) Mito-DCHO and (b) Mito-DCHO-Cys in DMSO-d$_6$.

11. ESI-MS spectra of Mito-DCHO, Mito-DCHO-Cys, Mito-SCHO and Mito-SCHO-Cys/Hcy

![ESI-MS spectra](image)

Fig. S11 (a) ESI-MS spectrum of Mito-DCHO. (ESI-MS m/z: [Mito-DCHO - Br]$^+$ C$_{52}$H$_{33}$N$_3$O$_4$P$^+$ calcd, 740.2718; found, 740.2702.) (b) ESI-MS spectrum of Mito-DCHO-Cys. (ESI-MS m/z: [Mito-DCHO-Cys - Br]$^+$ C$_{58}$H$_{49}$N$_3$O$_4$S$_2$P$^+$ calcd, 946.2902; found, 946.2924.)
Fig. S12 (a) ESI-MS spectrum of Mito-SCHO. (ESI-MS m/z: [Mito-SCHO - Br]^+ C_{43}H_{35}NOP^+ calcd, 612.2456; found, 612.2427.) (b) ESI-MS spectrum of Mito-SCHO-Cys. (ESI-MS m/z: [Mito-SCHO-Cys - Br]^+ C_{46}H_{40}N_{2}O_{2}SP^+ calcd, 715.2548; found, 715.2612.) (c) ESI-MS spectrum of Mito-SCHO-Hcy. (ESI-MS m/z: [Mito-SCHO-Hcy - Br]^+ C_{47}H_{42}N_{2}O_{2}SP^+ calcd, 729.2705; found, 729.2772.)

12. Two-photon absorption properties

Fig. S13 Two-photon absorption cross-section values of Mito-DCHO (0.1 mM) and
**Mito-DCHO** (0.1mM) with the addition of Cys (0.6 mM). Inset: The square relationship of two-photon excited fluorescence intensity \((I_{\text{out}})\) of **Mito-DCHO** and **Mito-DCHO** + Cys with input power \((I_{\text{in}} = 300-800 \text{ mW})\). Excitation was carried out at 760 nm and 720 nm, respectively.

13. Cytotoxicity Assays

![Cytotoxicity Assays](image)

**Fig. S14** Cytotoxicity data of **Mito-DCHO** (Hela cells incubated for 24 h).

14. TPM imaging of **Mito-DCHO**’s mitochondria-targeted capability

![TPM imaging](image)

**Fig. S15** TPM images of **Mito-DCHO**’s mitochondrial location. HeLa cells were co-stained with **Mito-DCHO** (10 μM) and Mitotracker red (1 μM) for 30 min at 37 °C. From left to right: (a) \(\lambda_{\text{em}} = 500-540 \text{ nm} \) (**Mito-DCHO**, \(\lambda_{\text{ex}} = 720 \text{ nm}\)) (b) \(\lambda_{\text{em}} = 580-600 \text{ nm} \) (Mitotracker red, \(\lambda_{\text{ex}} = 579 \text{ nm}\)) (c) Merge, overlay of (a) and (b). Insert: Intensity scatter plot of green and red channels. (d) Bright Field. Scale bar: 20 μm.
15. Dual-color imaging of **Mito-DCHO** to exogenous and endogenous Cys in cells

**Fig. S16** Dual-color images of **Mito-DCHO** responding to mitochondrial Cys in living HeLa cells. (A) HeLa cells were incubated with **Mito-DCHO** (10 μM) for 30 min. (B, C, D, E) HeLa cells were pretreated with NEM (1.0 mM) for 30 min and then incubated with blank, Cys, Hcy and GSH (60 μM) for 1 h, respectively, and finally incubated with **Mito-DCHO** (10μM) for 30 min. From left to right: Blue Channel: $\lambda_{em} = 420-460$ nm, Green Channel: $\lambda_{em} = 500-540$ nm, Merge and Bright Field. $\lambda_{ex} = 720$ nm. Scale bar: 20 μm.
16. Dual-color imaging of mitochondrial Cys under oxidative stress induced by H$_2$O$_2$

**Fig. S17** TPM images of Mito-DCHO in the absence and presence of H$_2$O$_2$ in living HeLa cells (1-4) and apoptosis analysis (5). (1-4) Cells were mediated by H$_2$O$_2$ (200 μM) for 0 and 30 min (A-B) respectively, subsequently incubated with Mito-DCHO (10 μM, 30 min), and imaged. From left to right: Blue Channel: $\lambda_{em} = 420$–460 nm, Green Channel: $\lambda_{em} = 500$–540 nm, Merge, Bright Field and Apoptosis. $\lambda_{ex} = 720$ nm. Scale bar: 20 μm. (5) HeLa Cells were mediated by H$_2$O$_2$ (200 μM) for 0 and 30 min (A5-B5) respectively, then cells were harvested and the percentage of cell apoptosis was analyzed by flow cytometer via Annexin V-FITC/PI Apoptosis Detection Kit. Apoptosis analysis: areas of B $-$ + , B $+$ + , B $-$ $-$ , B $+$ $-$ were corresponding necrotic, late apoptosis, viable, and early apoptosis. 
17. Dual-color imaging of mitochondrial oxidative stress levels under LPS-induced apoptosis

**Fig. S18** TPM images of mitochondrial oxidative stress levels under LPS-induced apoptosis utilizing probe **Mito-DCHO**. (A) Cells were incubated with **Mito-DCHO** (10 μM) for 30 min, and imaged. (B-F) Cells were mediated by LPS (5 μg/mL) for 0.5, 1.0, 1.5, 2.0 and 2.5 h respectively, subsequently incubated with **Mito-DCHO** (10 μM) for 30 min and imaged. From top to bottom: Green Channel: \( \lambda_{\text{em}} = 500-540 \text{ nm}, \lambda_{\text{ex}} = 720 \text{ nm} \); MTR: \( \lambda_{\text{em}} = 580-600 \text{ nm} \) (Mitotracker red, \( \lambda_{\text{ex}} = 579 \text{ nm} \)), Bright Field, Merge: overlay of Green Channel and MTR. Scale bar: 20 μm.

18. Ratiometric TPM images in zebrafish during LPS-induced apoptosis
Fig. S19 Ratiometric TPM images in zebrafish during LPS-induced apoptosis. (A-E) Zebrafishes were mediated by LPS (100 μg/mL) for 0, 0.5, 1, 1.5 and 2 h respectively, subsequently incubated with **Mito-DCHO** (20 μM) for 0.5 h, and imaged. From left to right: Blue Channel: λ_{em} = 420-460 nm; Green Channel: λ_{em} = 500-540 nm, λ_{ex} = 720 nm; Merge and ratio imaging of blue/green channel. Scale bar: 400 μm. (F) Fluorescence intensity ratios (I_{blue}/I_{green}) values in panels A-E. Data are expressed as mean ±SD of three parallel experiments.

19. NMR Spectra and mass original spectra

![NMR Spectra](image)

Fig. S20 ^1^H NMR spectra of compound 1 in CDCl3.
Fig. S21 $^1$H NMR spectra of compound 2 in CDCl$_3$.

Fig. S22 $^1$H NMR spectra of compound Mito-SCHO in DMSO-$_d_6$. 
Fig. S23 $^1$H NMR spectra of compound 3 in CDCl$_3$.

Fig. S24 $^1$H NMR spectra of compound 4 in CDCl$_3$. 
Fig. S25 $^1$H NMR spectra of Mito-DCHO in DMSO-$d_6$.

Fig. S26 $^{13}$C NMR spectra of compound 1 in CDCl$_3$. 
Fig. S27 $^{13}$C NMR spectra of compound 2 in CDCl$_3$.

Fig. S28 $^{13}$C NMR spectra of compound Mito-SCHO in DMSO-$d_6$. 
Fig. S29 $^{13}$C NMR spectra of 3 in CDCl$_3$.

Fig. S30 $^{13}$C NMR spectra of compound 4 in CDCl$_3$. 
Fig. S31 $^{13}$C NMR spectra of Mito-DCHO in DMSO-$d_6$.

Fig. S32 ESI-MS mass original spectra of Mito-SCHO.
**Fig. S33** ESI-MS mass original spectra of Mito-DCHO.

**Fig. S34** ESI-MS mass original spectra of Mito-SCHO-Cys.
**Fig. S35** ESI-MS mass original spectra of Mito-SCHO-Hcy.

**Fig. S36** ESI-MS mass original spectra of Mito-DCHO-Cys.