Supporting Information

Near-infrared fluorescence probe for in situ detection of superoxide anion and hydrogen polysulfides in mitochondrial oxidative stress

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1. General Methods

Materials: The stock solutions of Hcy-Mito, Hcy-Biot were solute in dimethyl sulfoxide (DMSO) and maintained in refrigerator at 4 ^{o}C. The commercial reagents were used according to the specification. H_2S_n were derived from H_2S and S_8.[1,2] KO_2 was prepared in twice-distilled water. CysSSH was generated by 0.5 mM Cys and 0.5 mM NaHS in the presence of 0.5 mM P-NONOate in 10 mM Tris-HCl buffer (pH 7.4) at 25 ^{o}C for 15 min.[3] $O_2^{\bullet-}$ was created by the enzymatic reaction of xanthine/xanthineoxidase (XA/XO; 6.0 μM/3 mU) at 25 ^{o}C for 5 min.[4,5] Methyl linoleate (MeLH) and 2,2'-azobis-(2,4-dimethyl)valeronitrile (AMVN) were used to produce MeLOOH.[6,7] GSNO was generated from GSH.[8] H_2O_2 was determined at 240 nm (ε= 43.6 $M^{-1}cm^{-1}$). OCl^- was standardized at pH = 12 ($\varepsilon_{292\ nm}$ = 350 $M^{-1}cm^{-1}$).[9] NO is generated in form of 3-(Aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5, 22 μM/mL). ·OH was generated by Fenton reaction between Fe^{II} (EDTA) and H_2O_2 quantitively, and Fe^{II} (EDTA) concentrations represented ·OH concentrations. Tert-butylhydroperoxide (*t*-BuOOH) and cumene hydroperoxide (CuOOH) could also be used to induce ROS in biological systems.[10] Na_2S_2, Na_2S_4 and Cys-polysulfide were prepared using reported procedures.[11-14] All chemicals used in synthesis were analytical reagent grade, and were used as received. Ultrapure water was used throughout.

Instruments: Thin-layer chromatography (TLC) was performed on silica gel plates. Silica gel P60 (SiliCycle) was used for column chromatography (Hailang, Yantai) 200 - 300 mesh. Absorption spectra were collected on a Thermo Scientific NanoDrop 2000/2000C spectrophotometer. Fluorescence spectra were determined using a HORIBA Scientific Fluoromax-4 spectro fluorometer. All pH measurements were performed with a basic pH-Meter PH-3C digital pH-meter (Lei Ci Device Works, Shanghai) with a combined glass-calomel electrode. ^{1}H NMR and ^{13}C NMR spectra were recorded employing a Bruker AVANCE IIITM 500 spectrometer. Fluorescent images of cells were acquired on an Olympus Fluo View FV1000 laser-scanning microscope with an objective lens (× 40). BALB/c mice images were collected by Bruker In-vivo Imaging System and BALB/c mice bearing murine sarcoma S180 tumor xenograft images were captured by Xenogen IVIS Spectrum Pre-clinical In Vivo Imaging System. BALB/c tumor-bearing mice S180 sarcoma tissue pathological sections were imaged by Motic BA400 Biological Research Compound Infinity Binocular Microscope.

Spectrophotometric Experiments: Absorption spectra were obtained with 1.0-cm cuvette cells. The probes Hcy-Mito and Hcy-Biot were added to a 10.0-mL color comparison tube. After dilution to 0.5 μM with 5 mM HEPES buffer, different concentrations of H_2S_n and KO_2 were added. The mixture was incubated at 37 ^{o}C for 5 min before measurement. Fluorescence spectra were obtained with a 1.0-cm quartz cells by Xenon lamp. The probe Hcy-Mito or Hcy-Biot was added to a 10.0-mL color comparison tube, respectively. After diluted to 10 μM with 10 mM HEPES buffer, different concentrations of H_2S_n were added. The mixture was incubated at 37 ^{o}C for 2 min before measurement.

Cell Culture: Mouse macrophage cell line (RAW264.7) and human umbilical vein epithelial cells (HUVECs) were obtained from the Committee on Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). HUVECs cells were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM, 4.5 g of glucose/L) supplemented with 10% Fetal Bovine Serum (FBS). Cultures were maintained at 37 °C under a humidified atmosphere containing 5% CO_2/95% air. Cells were plated on 25-Petri dishes and allowed to adhere for 24 hours before imaging. The cells were sub-cultured by scraping and seeding on 18 mm glass coverslips in

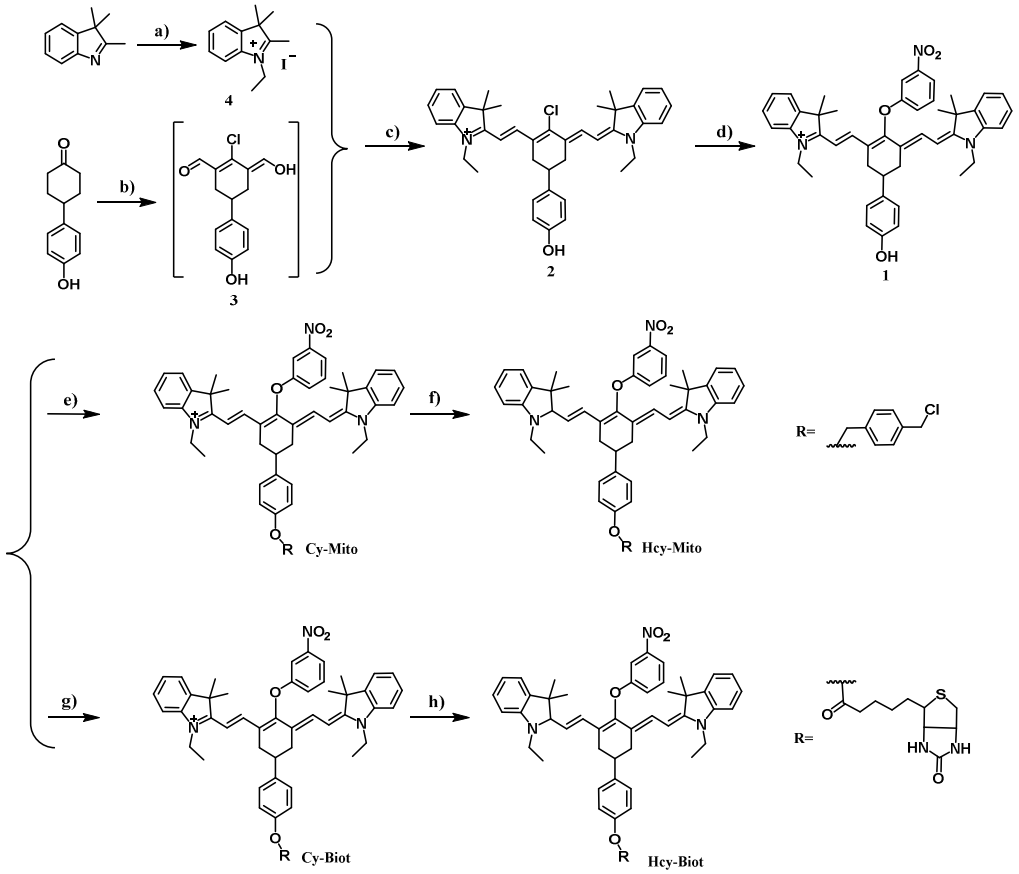
culture dish.

Confocal Imaging: The fluorescence images of RAW264.7 cells and HUVECs cells were taken by a confocal laser scanning microscope (Japan Olympus Co., Ltd) with an objective lens (× 40). Excitation wavelength of RAW264.7 cells and HUVECs cells was 730 nm, and the emission was collected from 750 nm to 800 nm. In the case of mitochondria and nucleus staining, excitation wavelengths were 488 nm and 405 nm, and the emission was collected from 500 nm to 580 nm and 425 nm to 500 nm, respectively.

Flow Cytometry Analysis: The cells were cultured at 2.0×10^5 cells/well in 6-well plates, and then treated with probes as described in the paper. After harvest, cells were washed and suspended in fresh complete medium and analyzed by flow cytometry. Excitation wavelength was 633 nm. The collected wavelengths were 750-810 nm.

2. Synthetic Procedures and Characterization Details

Scheme S1. Synthetic Approaches of Hcy-Mito and Hcy-Biot



a) Iodoethane, acetonitrile, refluxed for 12 h, 90%; b) DMF, CH_2Cl_2, $POCl_3$, 45 °C, 3 h, 85%; c) n-Butyl alcohol:benzene = 7:3 (v/v), refluxed, 3 h, 70%; d) NaH, 3-nitrophenol, DMF, 25 ^oC, 24 h, 50%; e) NaH, p-dichloroxylene, DMF, 25 ^oC, 24 h, 65%; f) Ethanol, $NaBH_4$, 0 ^oC, 10 min, 80%; g) Biotin, DMF, EDCI, DMAP, HOBt, 25 ^oC, 24 h, 69%; h) Ethanol, $NaBH_4$, 0 ^oC, 10 min, 82%.

Synthesis of N-ethyl-2,3,3-trimethylindolinium Iodide 4: 2, 3, 3-trimethyl-3H-indolenine (12 g, 75 mmol) and iodoethane (11.5 g, 75 mmol) were mixed in 40 mL anhydrous acetonitrile in 250 mL round flask, then the mixture was refluxed for 12 h, then stopped heating and cooled down. The precipitate was filtered through a buchner funnel, and the solid product was washed by diethyl ether and dried in vacuum to afford pink product (10.9 g, yield: 90%). ^{1}H NMR (DMSO-d_6, 500 MHz) δ(ppm): 8.04-8.02 (t, 1H), 7.91-7.89 (t, 1H), 7.66-7.62 (m, 2H), 4.57-4.53 (m, 2H), 2.92 (s, 3H), 1.58 (s, 6H), 1.49-1.46 (t, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 196.5, 142.4, 141.1, 129.4, 124.1, 115.8, 54.6, 43.8, 40.1, 22.4, 14.9, 13.3. LC-MS (ESI$^+$): m/z $C_{13}H_{18}N^+$ calcd. 188.1434, found [M^+] 188.1435.

Synthesis of Compound 3: A solution of 40 mL of anhydrous N, N-dimethylformamide (DMF) and 40 mL of anhydrous CH_2Cl_2 was placed in a 250 mL round-bottom flask, chilling the solution to -10 °C and then stirring for 20 min. Phosphorus oxychloride (37 mL), with 35 mL of anhydrous CH_2Cl_2 was dropwise added into above solution through a constant pressure drop of liquid funnel. 4-(4-Hydroxyphenyl)cyclohexanone (10 g, 52.6 mmol) was added into the mixture in batches, the solution changed from colorless into yellow immediately. Then the solution was slowly heated to 45 ^{o}C for 3 h, then cooled down, poured into a lot of ice, and allowed to stand overnight. The yellow solid was collected through a buchner funnel and dried in vacuum (12.9 g, yield: 85%). ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.22 (s, 1H), 8.12 (s, 1H), 8.08 (s, 1H), 7.65-7.34 (m, 2H), 7.14 (s, 1H), 6.69-6.64 (m, 2H), 2.38-2.71 (m, 1H), 2.53-2.45 (m, 2H), 2.28-2.26 (m, 2H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 191.8, 162.9, 155.7, 148.5, 145.7, 142.4, 131.2, 127.6, 127.5, 117.8, 115.1, 37.2, 31.7, 30.6. LC-MS (ESI$^-$): m/z $C_{14}H_{13}ClO_3$ calcd. 264.0553, found [M-H]$^-$ 263.0481.

Synthesis of Compound 2: Compound **4** (0.84 g, 2 mmol) and **3**（0.26 g, 1 mmol）were resolved in 100 mL mixed solution of *n*-butyl alcohol and benzene (7:3, v/v) in 250 mL round flask, refluxed for 3 h, dried in vacuum, to obtain green solid. The crude product was purified by silica gel chromatography using EtOAc/CH_3OH (8:1, v/v) as eluent to afford compound **2** as green solid (0.682 g, 70% yield). ^{1}H NMR ($CDCl_3$-d_1, 500 MHz) δ (ppm): 8.43-8.40 (d, 2H), 7.41-7.39 (m, 4H), 7.28-7.15 (m, 8H), 6.09-6.07 (d, 2H), 5.01 (s, 1H), 4.14-4.15 (m, 4H), 3.01-2.97 (m, 4H), 2.68-2.63 (m, 1H), 2.04 (m, 6H), 1.79 (m, 6H), 1.42-1.39 (m, 3H), 1.27-1.24 (m, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 172.2, 171.2, 156.1, 150.3, 144.6, 141.6, 141.2, 134.9, 128.9, 127.9, 126.8, 125.5, 122.3, 116.5, 110.9, 100.6, 60.4, 49.5, 39.9, 38.0, 34.2, 28.1, 21.1, 14.2, 12.5. LC-MS (ESI$^+$): m/z $C_{40}H_{44}ClN_2O^+$ calcd. 603.3137, found [M^+] 603.3137.

Synthesis of Compound 1: 3-nitrophenol (0.696 g, 5.00 mmol) and NaH (60% in mineral oil) (0.208 g, 5.00 mmol) were dissolved in anhydrous DMF (30 mL). The mixture was stirred at 25 ^{o}C for 15 min under Ar atmosphere. Then, the compound **2** (0.500 g, 0.830 mmol) was introduced into the above mixture. The reaction mixture was further stirred for 24 h at 25 ^{o}C. The solvent was removed under reduced pressure, and then the crude product was purified by silica gel chromatography using EtOAc/CH_3OH (4:1, v/v) as eluent to afford compound **1** as green solid (0.293 g, 50% yield). ^{1}H NMR ((DMSO-d_6, 500 MHz) δ (ppm): 7.79-7.94 (m, 4H), 7.69-7.62 (m, 2H), 7.40-7.20 (m, 10H), 6.84-6.82 (m, 2H), 6.21-6.18 (d, 2H), 4.65 (s, 1H), 4.12-4.11 (m, 4H), 3.56 (m, 1H), 3.30 (s, 6H), 2.99 (s, 6H), 2.85 (m, 4H), 1.37-1.34 (m, 3H), 1.31-1.28 (m, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 172.1, 163.4, 155.9, 141.6, 141.3, 139.1, 129.5, 128.4, 127.9, 125.1, 122.1, 121.6, 120.8, 118.2, 115.0, 115.01, 114.8, 113.5, 110.6, 110.3, 109.5, 107.3,

106.2, 99.8, 54.7, 53.6, 49.8, 48.9, 48.1, 47.2, 35.5, 31.6, 29.9, 29.3, 20.6, 11.0. LC-MS (ESI^+): m/z $C_{46}H_{48}N_3O_4^+$ calcd. 706.3639, found [M^+] 706.3640.

Synthesis of Hcy-Mito: Compound 1 (0.282 g, 0.40 mmol) and NaH (60% in mineral oil) (0.016 g, 0.40 mmol) were dissolved in 30 mL anhydrous DMF. The mixture was stirred at 25 ^oC for 30 min under argon atmosphere. Then *p*-dichloroxylene (0.350 g, 2.00 mmol) was introduced into theabove mixture. The reaction mixture was further stirred for 24 h at 25 ^oC. After removed solvent under reduced pressure, the crude product was purified by silica gel chromatography using CH_2Cl_2:MeOH (15:1, v/v) as eluent to afford Cy-Mito as green solid (0.219 g, 65%). ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 7.95-7.92 (m, 1H), 7.75-7.73 (m, 1H), 7.56-7.20 (m, 13H), 7.07 (m, 1H), 6.87-6.85 (m, 2H), 6.71 (m, 1H), 6.53-6.50 (m, 1H), 5.45 (s, 2H), 5.24-5.20 (d, 2H), 5.15 (s, 2H), 4.69 (s, 2H), 4.50-4.43 (m, 4H), 3.10 (m, 1H), 2.54-2.50 (m, 4H), 1.87 (s, 6H), 1.43 (s, 6H), 1.41-1.39 (m, 3H), 1.27-1.23(m, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 178.0, 175.9, 157.8, 154.4, 148.7, 141.6, 141.4, 135.7, 135.3, 129.7, 129.1, 128.9, 127.0, 126.3, 122.3, 122.2, 114.6, 112.6, 105.3, 97.3, 70.9, 50.1, 48.5, 45.4, 43.1, 38.4, 34.2, 28.2, 15.3, 13.6. LC-MS (ESI^+): m/z $C_{54}H_{55}ClN_3O_4^+$ calcd. 844.3876, found [M^+] 844.3875. Under Ar condition, Cy-Mito (0.228 g, 0.27 mmol) was dissolved in 10 mL ethanol. And then 1.5 equiv. $NaBH_4$ aqueous solution (1 mL) was dropwise added into the reaction system at 0 °C. After 10 min, the color of the mixture changed from green to yellow. Then added 50 mL CH_2Cl_2 and washed the mixture with saturated KI solution (100 mL × 3, bubbled with Ar to remove oxygen). Organic phase was evaporated by rotary evaporator, and the residues were purified by silica chromatography eluted with CH_2Cl_2 to give a brown solid (0.182 g, 80%). ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 7.81 (m, 1H), 7.67 (m, 1H), 7.40-7.25 (m, 7H), 7.09-7.00 (m, 2H), 6.80-6.72 (m, 7H), 6.56-6.53 (m, 2H), 6.02 (d, 2H), 5.76 (t, 2H), 5.14 (s, 2H), 4.70 (s, 2H), 4.18-4.06 (q, 4H), 3.42-3.39 (m, 1H), 3.15 (m, 1H), 2.72-2.62 (m, 2H), 2.53-2.50 (m, 2H), 1.80 (s, 6H), 1.51 (s, 6H), 1.33-1.28 (m, 3H), 1.15-1.10 (m, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 173.8, 157.2, 154.5, 149.1, 148.6, 130.1, 129.0, 128.8, 127.1, 126.4, 122.3, 120.8, 117.8, 114.1, 112.8, 109.9, 104.8, 97.5, 70.8, 50.3, 46.7, 45.7, 45.5, 35.8, 33.8, 28.1, 14.2, 13.5. LC-MS (ESI^+): m/z $C_{54}H_{56}ClN_3O_4$ calcd. 845.3959, found $[M+H]^+$ 846.4030.

Synthesis of Hcy-Biot: Biotin (0.122 g, 0.50 mmol) and 15 mL anhydrous DMF were added into 100 mL round-bottom flask, followed by addition of EDCI (0.115 g, 0.60 mmol), DMAP (0.061 g, 0.50 mmol), and HOBt (catalytic). The mixture was stirred for 30 min at 25 ^oC. Compound 1 (0.07 g, 0.1 mmol) was dissolved in 5 mL anhydrous DMF, and added slowly to the mixture. The reaction was lasted for 24 h, then diluted with ultrapure water (50 mL) and extracted with ethyl acetate (100 mL × 2). Then organic phase was washed with saturated KI solution (100 mL × 2). The organic layer was dried over by anhydrous $MgSO_4$. Concentrated and purified by column chromatography using CH_2Cl_2:MeOH (15:1, v/v) as eluent to afford Cy-Biot as green solid (0.063 g, 69%). ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 8.10 (m, 1H), 7.99-7.98 (m, 1H), 7.86-7.84 (m, 1H), 7.56-7.50 (m, 8H), 7.42-7.05 (m, 5H), 6.07-6.04 (d, 2H), 5.57 (s, 2H), 5.33-5.32 (m, 2H), 4.54 (m, 1H), 4.30-4.13 (m, 5H), 3.22-3.21 (m, 3H), 2.93 (m, 1H), 2.76-2.63 (m, 6H), 2.01-2.00 (m, 2H), 1.64-1.61 (m, 8H), 1.35-1.30 (m, 9H), 1.26-1.25 (m, 5H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 175.8, 175.7, 172.6, 159.6, 150.7, 149.5, 141.5, 141.3, 141.0, 130.1, 130.0, 129.9, 129.7, 128.9, 128.8, 128.4, 128.1, 125.5, 122.2, 120.8, 117.3, 110.7, 106.7, 98.4, 62.0, 60.1, 55.3, 50.4, 49.2, 43.4, 43.2, 39.7, 35.9, 33.6, 31.9, 29.3, 28.4, 28.3, 27.8, 27.7, 27.2, 25.5, 14.1, 12.5. LC-MS (ESI^+): m/z $C_{56}H_{62}N_5O_6S^+$ calcd. 932.4415, found [M^+] 932.4415.

Under Ar condition, Cy-Biot (0.251 g, 0.27 mmol) was dissolved in 10 mL ethanol. Then 1.5 equiv. $NaBH_4$ aqueous solution (1 mL) was dropwise added into the reaction system at 0 ℃. And 10 min later, the mixture changed its color from green to yellow. After added 50 mL CH_2Cl_2, the mixture was washed with saturated KI solution (100 mL × 3, bubbled with Ar to remove oxygen). Evaporated solvent by rotary evaporator, and the residues were purified by silica chromatography eluted with CH_2Cl_2 to give a brown solid (0.201 g, 82%). ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 7.69-7.64 (m, 1H), 7.53 (m, 1H), 7.40-7.01 (m, 9H), 6.78-6.71 (m, 3H), 6.53-6.45 (m, 2H), 5.91-5.89 (m, 2H), 5.70-5.69 (m, 2H), 5.19-5.18 (d, 2H), 4.62-4.58 (m, 1H), 4.30-4.13 (m, 5H), 3.51-3.40 (m, 3H), 3.35-2.91(m, 2H), 2.59-2.56 (t, 2H), 2.43-2.26 (m, 6H), 1.80-1.73 (m, 8H), 1.42 (s, 6H), 1.30-1.10 (m, 8H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 175.5, 173.3, 170.8, 163.4, 149.8, 149.5, 147.8, 141.0, 140.4, 138.1, 128.8, 128.6, 127.1, 127.0, 126.7, 126.3, 122.3, 122.0, 121.0, 120.6, 111.5, 110.3, 97.8, 77.2, 62.1, 60.1, 55.4, 50.1, 48.8, 45.5, 44.1, 39.6, 35.6, 33.5, 32.0, 31.8, 29.3, 28.4, 28.2, 27.8, 27.2, 25.4, 14.4, 12.8. LC-MS (ESI$^+$): m/z $C_{56}H_{63}N_5O_6S$ calcd. 933.4499, found $[M+H]^+$ 934.4570.

3. pH Effects on Probes

Standard fluorescence pH titrations were performed in 10 mM HEPES solution at a concentration of 10 μM (Cy-Mito, Cy-Mito-NH_2 and Cy-Biot, Cy-Biot-NH_2). As shown in Figure S1, the pH of the mediums hardly had effect on fluorescence intensity within the range from 3.0 to 8.4，which meant that the probe would work well under physiological conditions (pH = 7.40).

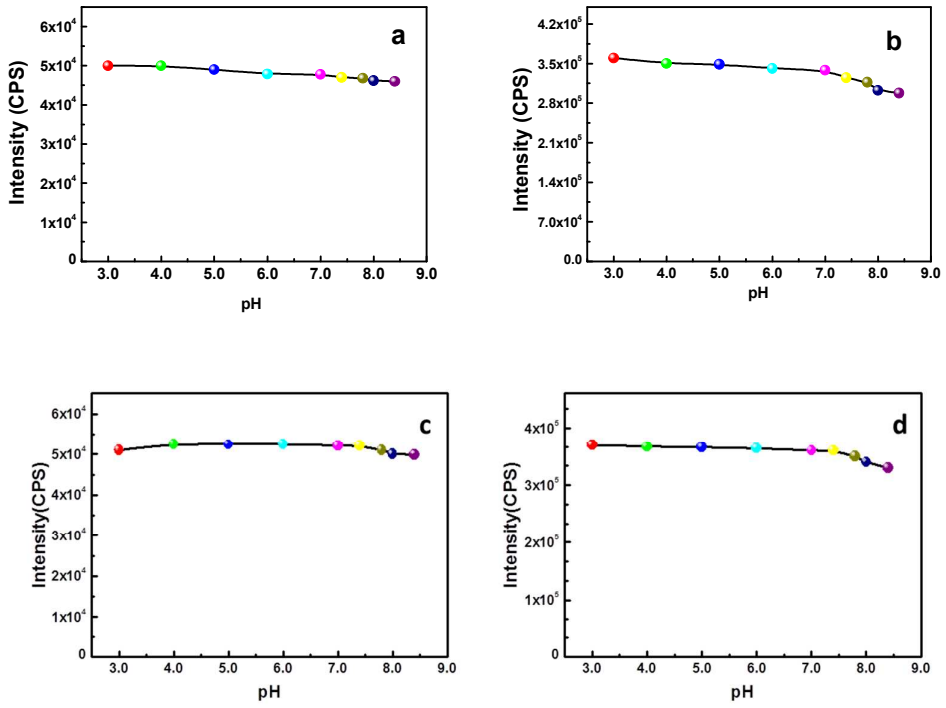


Figure S1. Effect of pH values. a) The fluorescence emission changes at 780 nm with the pH titration curve of Cy-Mito (10 μM). b) The fluorescence emission changes at 780 nm with the pH titration curve of Cy-Mito-NH_2 (10 μM). c) The fluorescence emission changes at 780 nm with the pH titration curve of Cy-Biot (10 μM). d) The fluorescence emission changes at 780 nm with the

pH titration curve of Cy-Biot-NH_2 (10 μM). pH: 3.0, 4.0, 5.0, 6.0, 7.0, 7.4, 7.8, 8.0, 8.4 (10 mM HEPES buffer solution).

4. Absorption Spectra of Hcy-Mito

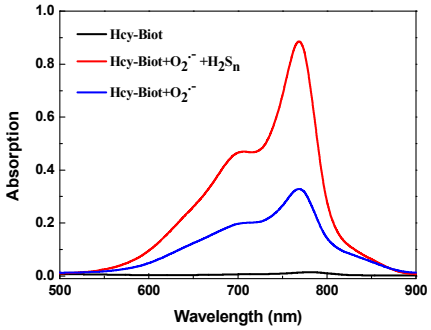


Figure S2. Absorption spectra of Hcy-Mito, Hcy-Mito + $O_2^{\bullet-}$ and Hcy-Mito + $O_2^{\bullet-}$ + H_2S_n. The black curve was the absorption spectrum of Hcy-Mito. The red curve was recorded after treatment with $O_2^{\bullet-}$. The blue curve was recorded after treatment with H_2S_n.

5. Reaction Kinetics and Selectivity to $O_2^{\bullet-}$ and H_2S_n

Owing to the rapid metabolism and unstable properties of $O_2^{\bullet-}$ and H_2S_n in biological systems, fast response time plays a crucial role in real time detection. The immediate response also could offer availability for direct detection without the cumbersome pretreatment processes. To test the above-mentioned feature, we further evaluated the probe's reactivity by time-dependent fluorescence spectra in HEPES buffer (10 mM, pH 7.4) at 37 °C for 120 min. As demonstrated in Figure S3, the fluorescence signal of Hcy-Mito could respond to $O_2^{\bullet-}$ within 10 min. After the addition of H_2S_n, the probe would give another increase in fluorescence within 10 min. The result revealed that our probe could provide fast response for the in situ detection of $O_2^{\bullet-}$ and H_2S_n. Our probe also had a good photostability in the tests. We next examined the response of Hcy-Mito towards endogenous $O_2^{\bullet-}$ and H_2S_n under simulated physiological conditions. $O_2^{\bullet-}$ was generated by enzymatic reaction of xanthine (XA) and xanthine oxidase (XO). H_2S_n were derived from cystathionine γ-lyase (CSE)-mediated cysteine metabolism. As expected, Hcy-Mito could capture enzymatically produced $O_2^{\bullet-}$ and H_2S_n under simulated physiological conditions. Moreover, the CSE inhibitor, DL-propargylglycine (PAG), would severely suppress the fluorescence emission indicating the low level of H_2S_n. The results illustrated that our probe could selectively respond to $O_2^{\bullet-}$ and H_2S_n. The fast reaction kinetics would be conducive to the real-time detection in cells and in vivo.

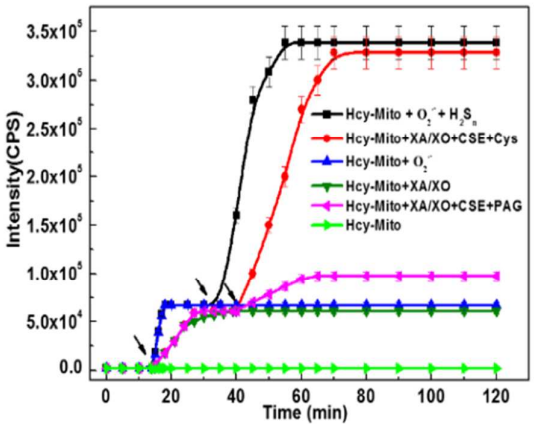


Figure S3. Time-dependent fluorescence spectra of probe Hcy-Mito (10 μM) towards $O_2^{\bullet-}$ and H_2S_n during 120 min. a) Hcy-Mito + $O_2^{\bullet-}$ (25 μM) + H_2S_n (100 μM); b) Hcy-Mito + xanthine/xanthine oxidase (6.0 μM/3 mU) + CSE (50 μg/mL) + cystine (1.25 mM); c) Hcy-Mito + $O_2^{\bullet-}$ (25 μM); d) Hcy-Mito + xanthine/xanthine oxidase(6.0 μM/3 mU); e) Hcy-Mito + xanthine/xanthine oxidase (6.0 μM/3 mU) + CSE (50 μg/mL) + PAG (100 μM) + cystine (1.25 mM); f) Free Hcy-Mito. Data were acquired in HEPES (10 mM, pH 7.4) at 37 °C. Fluorescence signals with λ_{ex} = 730 nm, λ_{em} = 780 nm.

To verify the fluorescent response to other biological analytes, we tested the selectivity of Hcy-Mito against physiological relevant ROS and reactive nitrogen species (RNS) in HEPES solution (10 mM, pH 7.4). Compared to other ROS and RNS, Hcy-Mito offered remarkable fluorescent response for $O_2^{\bullet-}$. Figure S4a demonstrated that H_2O_2, methyl linoleate hydroperoxide, cumene hydroperoxide, *tert*-butyl hydroperoxide, NO, ClO^- and S-nitrosoglutathione (GSNO) caused almost no response in emission signal for 20 min incubation. $^{\bullet}OH$ and $ONOO^-$ would trigger limited fluorescence response. However, the fluorescence increase was far weaker than that caused by $O_2^{\bullet-}$. The results showed that Hcy-Mito had good selectivity for $O_2^{\bullet-}$ detection over other biologically relevant ROS and RNS. The fluorescence responses of Cy-Mito to other RSS were also evaluated (Figure S4b). Cy-Mito (10 μM) could provide obviously fluorescence response to H_2S_n, such as H_2S_2, H_2S_4, H_2S_n (derived from H_2S and S_8). There was also obtained limit interference after addition of H_2S and cysteine hydropersulfide. However, no obvious changes in spectra were observed upon the addition of S_8, $PhCH_2S_4CH_2Ph$, Cys-polysulfide, GSH, Cys, Hcy, Cystine, and GSSG. The addition of ascorbic acid and α-tocopherol also caused no interferences. These results demonstrated that Cy-Mito was a highly selective fluorescent probe for H_2S_n detection over other RSS and physiologically relevant reduced species. Moreover, Cy-Mito had good fluorescence stability in HEPES buffer ranging from pH 4.0 to 9.0. Taken together, these kinetic and selectivity assays revealed that our probe could work well under physiological conditions for the investigation of $O_2^{\bullet-}$ and H_2S_n.

To verify whether there is fluorescence response to common metal ions and anions or not, Cy-Mito (10 μM) was treated with various analytes in HEPES buffer (pH 7.4, 10 mM). As shown in Figure S5, the K^+, Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Cl^-, Br^-, HSO^{3-}, SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, CO_3^{2-}, $H_2PO_4^{2-}$ did not trigger any fluorescence enhancement.

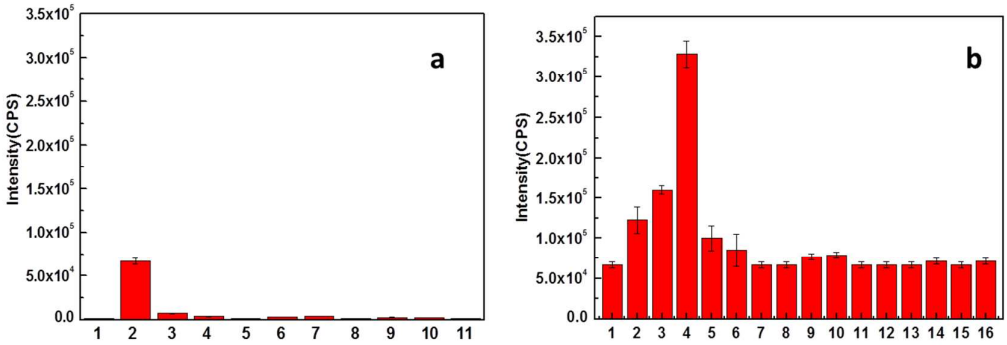


Figure S4. a) Fluorescence response of 10 μM Hcy-Mito to reactive oxygen and nitrogen species. 1, Blank; 2, $O_2^{\bullet-}$ (25 μM); 3, $^{\bullet}OH$ (25 μM); 4, H_2O_2 (100 μM); 5, methyl linoleate hydroperoxide (MeLOOH, 200 μM); 6, cumene hydroperoxide (CuOOH, 200 μM); 7, $ONOO^-$ (50 μM); 8, NO (200 μM); 9, *tert*-butyl hydroperoxide (*t*-BuOOH, 200 μM); 10, ClO^- (400 μM); 11, GSNO (20 μM). b) Selectivity of Cy-Mito toward H_2S_n together with various reactive sulfur species. 1, Blank; 2, H_2S_2 (Na_2S_2, 100 μM); 3, H_2S_4 (Na_2S_4, 100 μM); 4, H_2S_n (100 μM NaHS + 300 μM S_8); 5, H_2S (Na_2S, 100 μM); 6, cysteine hydropersulfide (100 μM); 7, S_8 (300 μM); 8, $PhCH_2S_4CH_2Ph$ (200 μM); 9, Cys-polysulfide (100 μM); 10, GSH (1 mM); 11, Cys (500 μM); 12, Hcy (500 μM); 13, cystine (500 μM); 14, GSSG (500 μM); 15, α-Tocopherol (50 μM); 16, ascorbic acid (50 μM). All data were acquired in HEPES (pH 7.4, 10 mM) at 37 °C (λ_{ex} = 730 nm, λ_{em} = 780 nm). All data were acquired in 10 mM HEPES (pH 7.4) at 37 °C after maintained 20 min (λ_{ex} = 730 nm, λ_{em} = 780 nm).

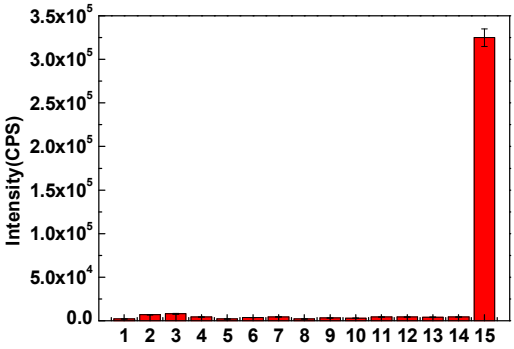


Figure S5. Fluorescence responses of Cy-Mito to various metal ions and anions. Legend: 1, K^+ (1 mM); 2, Na^+ (1 mM); 3, Ca^{2+} (1 mM); 4, Mg^{2+} (1 mM); 5, Zn^{2+} (1 mM); 6, Cu^{2+} (1 mM); 7, Cl^- (1 mM); 8, Br^- (1 mM); 9, HSO^{3-} (100 μM); 10, SO_4^{2-} (100 μM); 11, SO_3^{2-} (100 μM); 12, $S_2O_3^{2-}$ (100 μM); 13, CO_3^{2-} (100 μM); 14, $H_2PO_4^{2-}$ (100 μM); 15, H_2S_n (100 μM). Data were recorded in 10 mM HEPES buffer (pH=7.4, 10 mM) at 37 °C for 35 min. λ_{ex} = 730 nm, λ_{em} = 780 nm.

6. Cytotoxicity Assay

HUVECs cells were cultured in DMEM supplemented with 10% FBS in an atmosphere of 5% CO_2 and 95% air at 37 ^oC. The cells (8000/cell) were plated into 96-well plates and allowed to adhere for 24 hours. Subsequently, the cells were incubated with 0, 10, 20, 30, 40, 50, 60, 70 and 80 μM (final concentration) of Hcy-Mito, Cy-Mito, Hcy-Biot and Cy-Biot at 37 ^oC in an

atmosphere of 5% CO_2 and 95% air for 24 h. An untreated assay with DMEM was also performed under the same conditions. MTT solution (5.0 mg/mL in PBS, 20 μL) was added to each well, and 4 h later, the remaining MTT solution was carefully removed. In addition, DMSO (150 μL) was added to each well to dissolve the formazan crystals. The plate was shaken for 10 min and the absorbance was measured at 570 nm and 630 nm using a microplate reader (TECAN infinite M200pro). As shown in Figure S6, S7, S8, S9, when Hcy-Mito, Cy-Mito, Hcy-Biot and Cy-Biot were added, the concentration of 80 μM Hcy-Mito, Cy-Mito, Hcy-Biot and Cy-Biot were safe for the cell viability during 6 h window, and IC_{50} = 130, 125, 150, 152 μM, respectively.

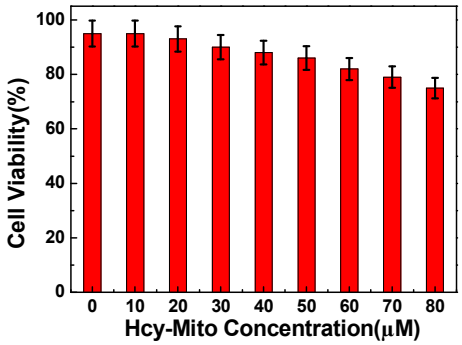


Figure S6. The 24 h cell viability for Hcy-Mito, the concentration of Hcy-Mito was 0, 10, 20, 30, 40, 50, 60, 70 and 80 μM.

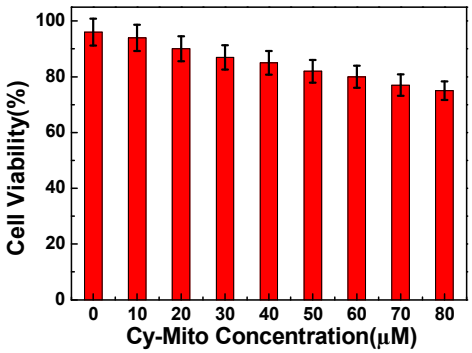


Figure S7. The 24 h cell viability for Cy-Mito, the concentration of Cy-Mito was 0, 10, 20, 30, 40, 50, 60, 70 and 80 μM.

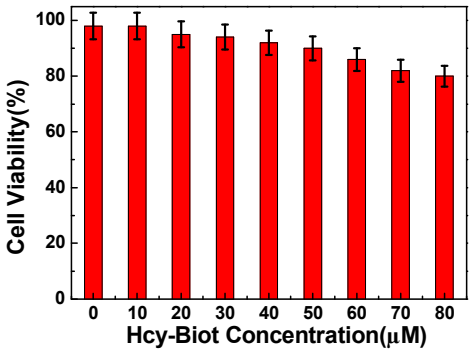


Figure S8. The 24 h cell viability for Hcy-Biot, the concentration of Hcy-Biot was 0, 10, 20, 30, 40, 50, 60, 70 and 80 μM.

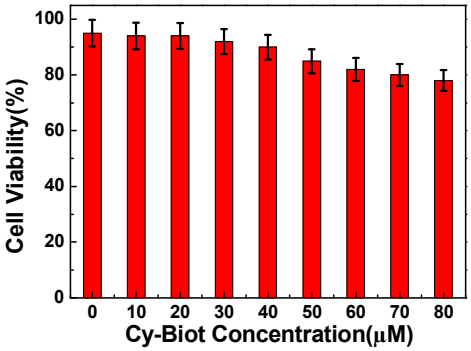


Figure S9. The 24 h cell viability for Cy-Biot, the concentration of Cy-Biot was 0, 10, 20, 30, 40, 50, 60, 70 and 80 μM.

7. The Average Fluorescence Intensities in Figure 2

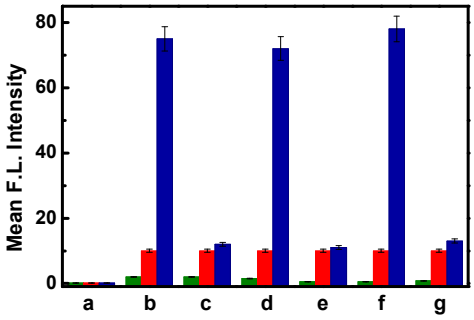


Figure S10. The average fluorescence intensity in Figure 2 correspondingly. The cell body regions in Figure 2a) – 2g) were selected as the regions of interest (ROI), and the average fluorescence intensity was determined via confocal laser-scanning microscopy. Data were normalized to controls and statistical analyses were performed with a two-tailed Student's *t*-test. *P < 0.05 (n = 3). Error bars are ± s.e.m.

8. Bright-field Images of Figure 2.

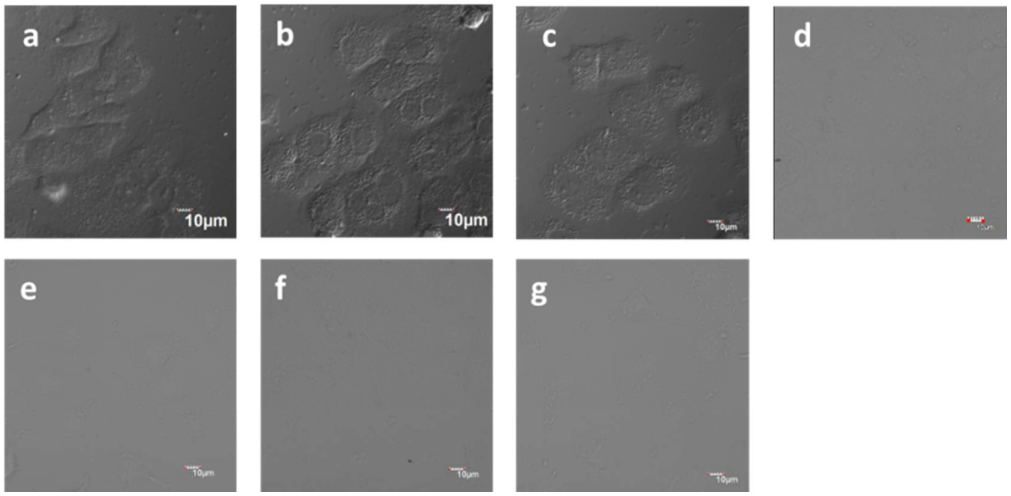


Figure S11. Bright-field images of Figure 2a) - 2g). Scale bar: 10 μm.

9. The Supplementary Cell Images of Figure 3.

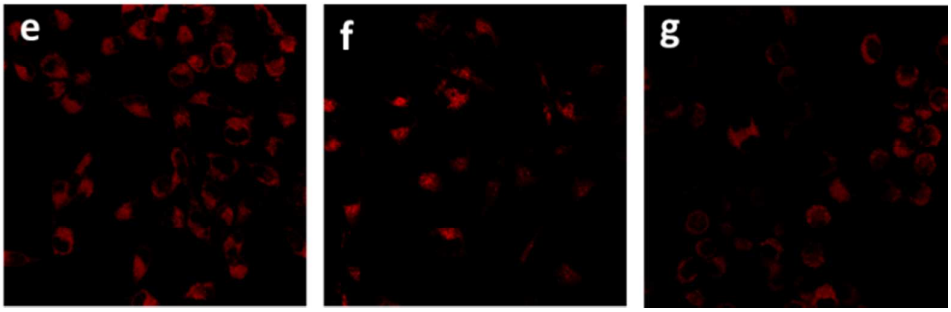


Figure S12. The cell images at the time point 60 min for Figure 3e) - 3g).

10. The Mean Fluorescence Intensity for Figure 3

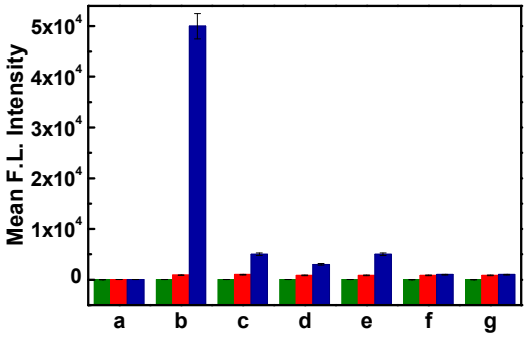


Figure S13. The mean fluorescence intensity of flow cytometric analyses in Figure 3.

11. Bright-field Images of Figure 3.

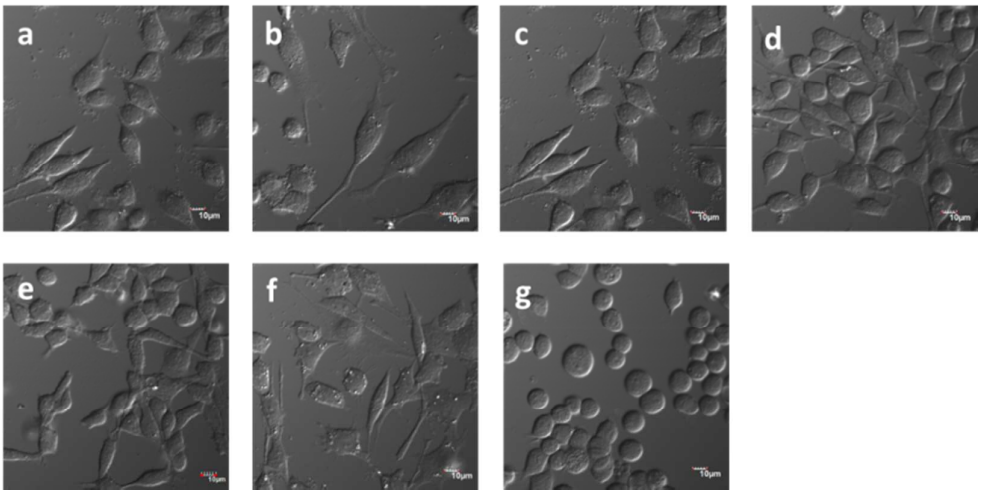


Figure S14. Bright-field images of Figure 3a) - 3g). Scale bar: 10 μm.

12. Sublocation of $O_2^{\bullet-}$ in Cells

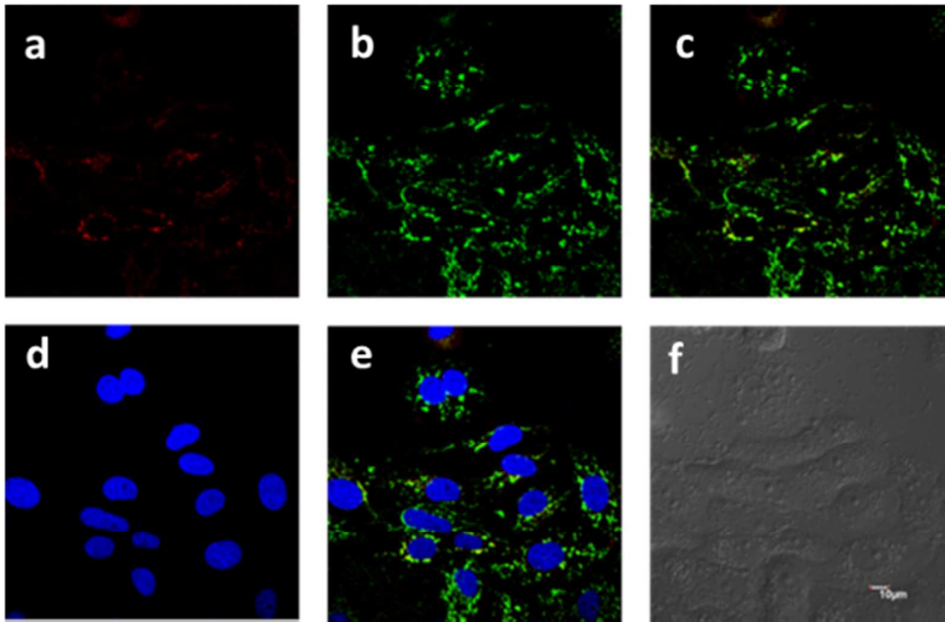


Figure S15. Mitochondrial multicolor colocalization in HUVECs cells with probe Hcy-Mito, MitoTracker® Green FM, and Hoechst 33342. a) HUVECs cells were treated with 50 μM paraquat for 8 h, and then incubated with Hcy-Mito 1 μM for 15 min. b) 1 μg/mL MitoTracker® Green FM for 15 min. c) Merged red and green channels. d) 1 μg/mL Hoechst 33342 for 30 min. e) Merged red, green, and blue channels. f) Bright field. Images displayed represent emission intensities collected in optical windows between 750 and 800 nm upon excitation at 730 nm for Hcy-Mito. Emission intensities collected in optical windows between 500 and 580 nm upon excitation at 488 nm for MitoTracker® Green FM. Emission intensities collected in optical windows between 425 and 500 nm upon excitation at 405 nm for Hoechst 33342.

13. Flow Cytometry Analysis for Mitochondria

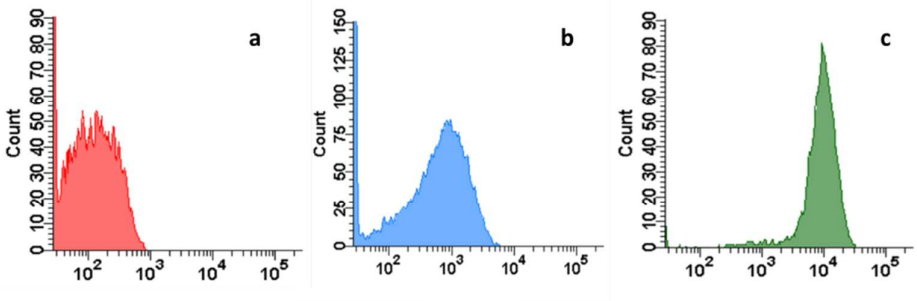
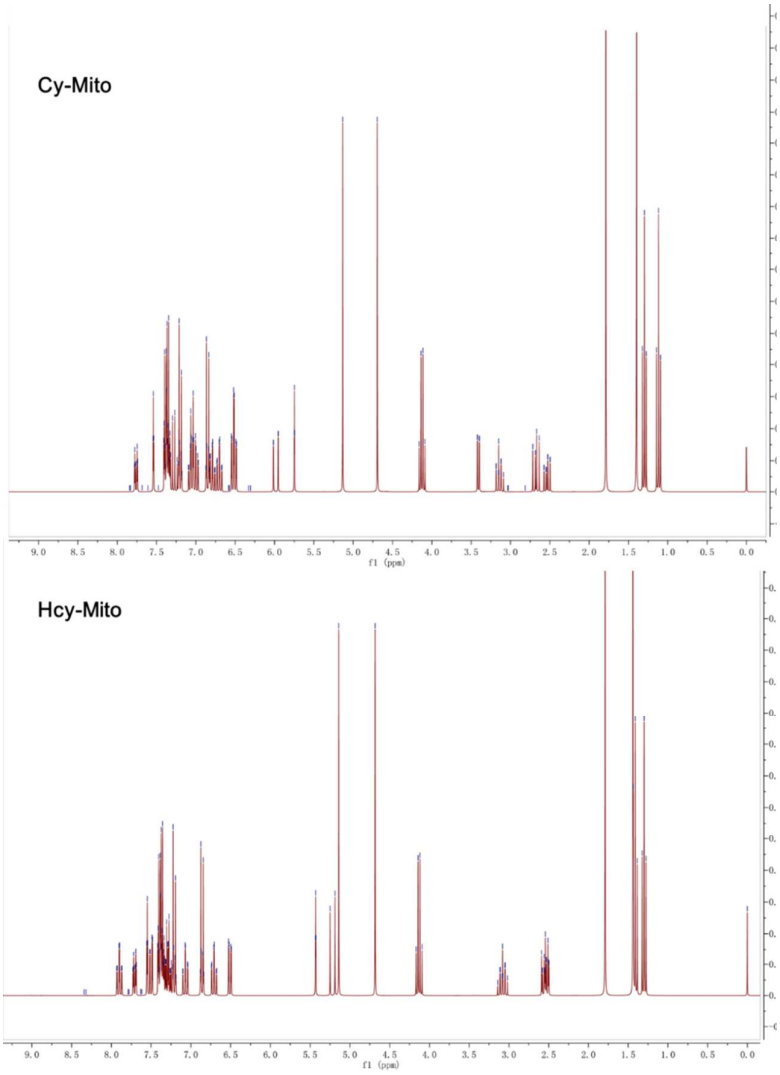
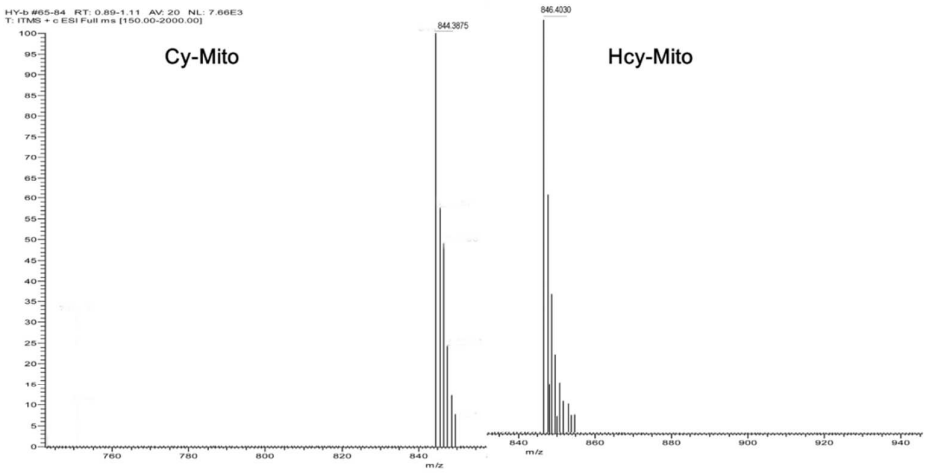
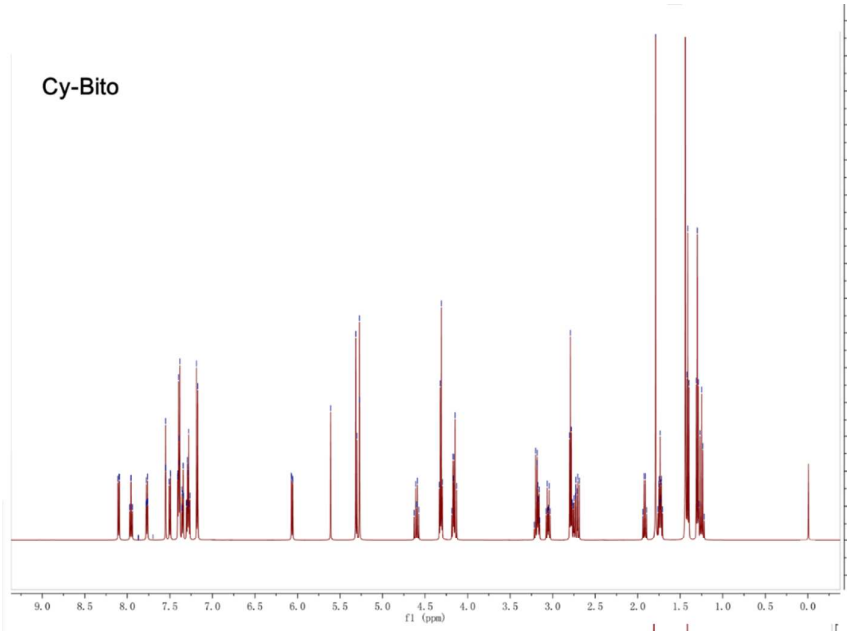


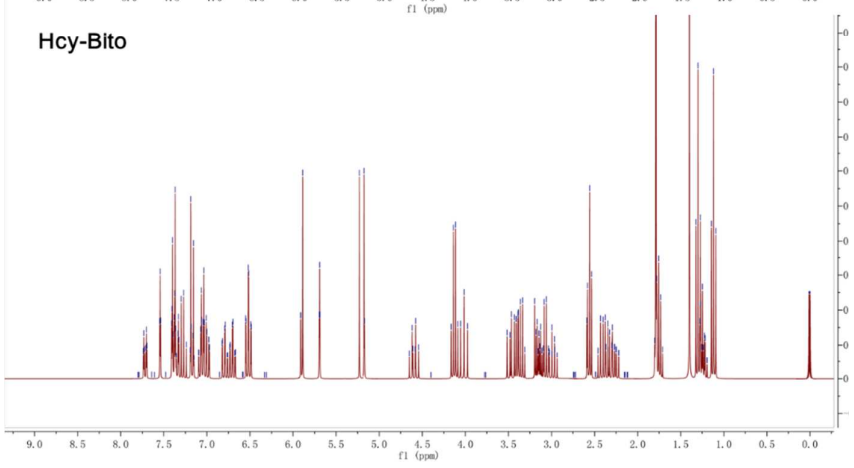
Figure S16. HUVECs cells were treated as follows, and then the mitochondria of these cells were extracted. a) Control. b) HUVECs cells were treated with 50 μM paraquat for 8 h, and then incubated with 1 μM Hcy-Mito for 15 min. c) HUVECs cells were treated with 50 μM paraquat for 8 h, incubated with 10 μM Hcy-Mito for 15 min, and then cultured for more 15 min.

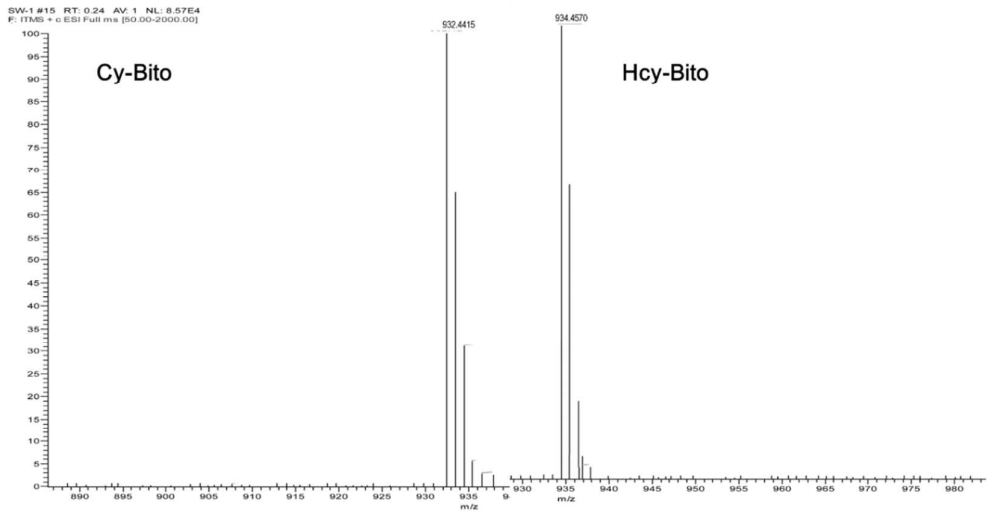
14. ^{1}H NMR and HRMS of Cy-Mito, Hcy-Mito, Cy-Biot and Hcy-Biot




Cy-Mito
844.3875
Hcy-Mito
846.4030
m/z


Cy-Bito
f1 (ppm)


Hcy-Bito
f1 (ppm)



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