Supporting Information

Illuminating Superoxide Anion and pH Enhancements in Apoptosis of Breast Cancer Cells Induced by Mitochondrial Hyperfusion Using a New Two-photon Fluorescence Probe

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1. Materials and reagents

All chemicals were purchased from Adamas Reagent, Ltd. (China) and Energy Chemica,l Ltd. (China), and analytical grade solvents were used without further purification. We prepared the reactive oxygen species (ROS) as follows. H_2O_2, hypochlorite (NaOCl) and tert-butyl hydroperoxide (TBHP) were used in aqueous solutions of 30%, 10% and 70%, respectively. Hydroxyl radicals (•OH) were generated by the reaction of 1 mM Fe^{2+} with 200 μM H_2O_2. Singlet oxygen ($^1O_2$) was prepared using the ClO^-/H_2O_2 system, andperoxynitrite was created from a stock solution of 10 mM in 0.3 M NaOH. Superoxide ($O_2^{\cdot-}$) was generated from KO_2 in DMSO solution.

2. Instruments

^{1}HNMR spectra were examined at 400 MHz using Bruker NMR spectrometers. The mass spectra were obtained using the Bruker maXis ultra-high resolution-TOF MS system. All one-photon (OP) fluorescence measurements were carried out at room temperature on an FLS-980 Edinburgh fluorescence spectrometer. The two-photon excited fluorescence spectra were measured using a Zeiss LSM 880 NLO.

One-photon fluorescent images were acquired on a Leica TCS SP5 confocal laser-scanning microscope with an objective lens (×63 and ×100) and Zeiss Airyscan (×100). The excitation wavelength was 405 nm (0.5 mW) and 488 nm (4.5 mW). Following incubation, the cells were washed three times with PBS and imaged. The TP images were acquired with a Zeiss LSM 880 NLO (25× water objective). The Ti:sapphire laser was used to excite the specimen at 800 nm, and the laser power was 70 mW.

3. Synthesis of probe CFT

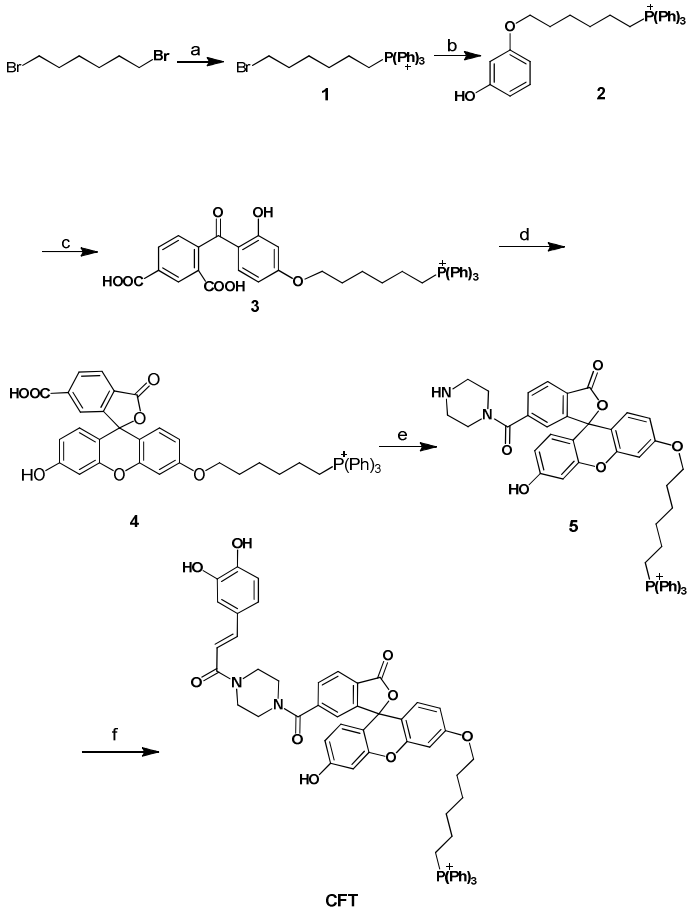

Figure S1. Synthesis of CFT; a) triarylphosphines, acetonitrile, 80°C; b) resorinol, $CsCO_3$, DMF, room temperature (r.t.); c) 1,2,4-benzenetricarboxylic anhydride, $AlCl_3$, cichloroethane, r.t.; d) resorinol, TFA, methanesulfonic acid, 0°C; e) 1,4-diazacyclohexane, DCC, 4-dimethylaminopyridine (DMAP), CH_2Cl_2, r.t.; f) EDC, HOBT, DMF, DCM, r.t.

Synthesis of Compound 1

1,6-dibromohexane (1.5 mL, 10 mmol) and triphenylphosphine (0.26 g, 1.0 mmol) were dissolved in acetonitrile (5 mL), and the mixture was stirred under reflux for 12 h. After the reaction was completed, the mixture was cooled to room temperature. The solvent was then centrifuged, and the residue was washed three times with water to yield compound 1 as a white solid. HRMS data, m/z calculated for $[C_{24}H_{27}BrP^+]$ 425.1034, found 425.1034.

Synthesis of Compound 2

Compound 1 (0.43 g, 1.0 mmol) and resorinol (1.1 g, 10 mmol) were first dissolved in DMF (10 mL), followed by the addition of Cs_2CO_3 (0.33 g, 1.0 mmol). The mixture was stirred at room temperature under nitrogen for 1 h.

The reaction mixture was extracted with dichloromethane (40 mL × 3). The combined dichloromethane fractions were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure.The residue was further separated by column chromatography (silica, petroleum ether:ethyl acetate = 20:1) to yield 2 as a yellow oil. HRMS data, m/z calculated for $[C_{30}H_{32}O_2P^+]$ 455.2140, found 455.2138.

Synthesis of Compound 3

Compound 3 was prepared by modified methods as reported in the literature.[1] The Friedel-Crafts acylation with 1,2,4-benzenetricarboxylic anhydride in the presence of $AlCl_3$ generated the desired benzophenone derivatives 3. HRMS data, m/z calculated for [$C_{39}H_{36}O_7P^+$], 647.2199 found 647.2230.

Synthesis of Compound 4

A mixture of compound 3 (0.065 g, 0.10 mmol) and resorcinol (0.022 g, 0.20 mmol) was added with 7 mL of TFA and cooled to 0°C. A total of 2.5 mL of methanesulfonic acid was slowly added to this solution, and the temperature was maintained at 0-2°C using an ice water bath. The reaction mixture was stirred at 0°C for 15 h and then poured onto 50 g of ice. The precipitated materials were collected by centrifugation and resuspended in water (50 mL) with the addition of triethylamine (pH=10). The resultant solution was heated to boiling and then slowly cooled to room temperature. It was then acidified with concentrated hydrochloric acid to pH=2 and extracted with ethyl acetate (3× 100 mL). The extract was washed with brine and dried over $MgSO_4$. Then, the extract was concentrated, and a tan, viscous oil was obtained, which could be solidified upon treatment with water (~100 mL). Filtration and drying in a vacuum dryer yielded an orange solid, which was purified using a home-made silica gel thin-layer (ethanol/aqueous= 10/1). HRMS data, m/z calculated for [$C_{45}H_{38}O_7P^+$], 721.2355 found 721.2402.^{1}HNMR (400 MHz, DMSO): δ 10.18 (d, 1H), 8.38 (m, 1H), 7.95 (s, 1H), 7.88 (m, 2H), 7.51 (m, 3H), 6.93 (s, 1H), 6.84 (m, 10 H), 6.28 (m, 4H), 6.11 (m, 2H), 4.35 (s, 1H), 3.54 (d, 2H), 2.67 (d, 2H), 1.99 (m, 2H), 1.73 (m, 2H), 1.45 (m, 2H), 1.07 (m, 2H). ^{13}CNMR (101 MHz, DMSO) δ 174.45, 162.82, 157.98, 157.25, 155.58, 152.00, 148.57, 146.66, 145.99, 141.36, 130.84, 130.13, 129.13, 126.83, 125.07, 122.29, 121.82, 121.09, 120.41, 116.95, 116.70, 115.65, 115.14, 113.34, 102.06, 73.99, 51.65, 25.79, 25.40, 24.95, 23.40, 22.55.

Synthesis of CFT

A solution of compound 4 (0.072 g, 0.1 mM) and 1,4-diazacyclohexane (0.086 g, 1.0 mM) was added to DCC (0.21 g, 1.0 mM), 4-dimethylaminopyridine (DMAP, 0.12 g, 1.0 mM) and 15 mL H_2Cl_2. The reaction mixture was stirred at 40°C for 15 h and then precipitated with 50 mL ice water. Filtration and drying in a vacuum dryer yielded a dark orange solid that recrystallized with ethanol. Crude product 5 was then obtained, and we performed the same synthesis procedure for CFT. Caffeic acid (0.18 g, 1.0 mmol) and product 5 were mixed into a flask containing DCC (0.21 g, 1.0 mM), 4-dimethylaminopyridine (DMAP, 0.12 g, 1.0 mM) and 15 mL H_2Cl_2. After the mixture was stirred at 40°C for 48 h, it was concentrated under vacuum. The residue was purified by preparative thin layer chromatography on silica gel GF_{254} using toluene/methyl alcohol/ethyl acetate/(4/0.5/2) as the eluent, and a yellow product was obtained.

HRMS data, m/z calculated for [$C_{58}H_{52}N_2O_9P^+$], 951.3410 found 951.3377.^{1}HNMR (400 MHz, DMSO): δ 0.97 (m, 2H), 1.49 (m, 2H), 1.60 (m, 2H), 1.70 (m, 2H), 2.5 (d, 2H), 3.79 (d, 2H), 6.18 (d, 1H), 6.32 (d, 2H), 6,53 (m, 4H), 6.69 (m, 5H), 6.82 (m, 5H), 6.95 (s, 2H), 7.21 (d, 1H), 7.78 (m, 10H), 7.96 (s, 1H). ^{13}CNMR (400 MHz, DMSO) δ 170.90, 169.34, 162.79, 159.70, 159.54, 158.78, 157.24, 152.11, 148.35, 147.42, 146.70, 145.82, 141.01, 137.38, 134.40, 131.97, 130.10, 129.12, 126.97, 122.25, 121.85, 120.95, 120.38, 118.28, 116.59, 114.99, 114.99, 113.92, 111.18, 110.57, 110.34, 106.52, 102.48, 102.33, 101.43, 83.20, 65.61, 49.04, 47.97, 40.88, 40.39, 29.48, 29.15, 25.80, 25.41, 24.93, 23.51.

4. Photophysical properties of CFT

The as-prepared compound displayed a maximal absorption band at approximately 480 nm (fluorescein moiety, ε= 2.2×10^5 M^{-1} cm^{-1}, 30 mM PBS buffer, pH 7.4). After adding 30 μM $O_2^{\bullet-}$ and increasing the pH from 7.4 to 9.0, an absorption peak appeared at 374 nm (caffeoyl moiety, ε= 6.9×10^5 M^{-1} cm^{-1}), and a higher molar extinction coefficient at 480 nm (ε= 8.8×10^5 M^{-1} cm^{-1}) was observed in PBS at ambient temperature (Fig. S2a). Upon excitation at 400 nm (Figure S2b), more intense blue fluorescence was observed in the presence of $O_2^{\bullet-}$. Figs. S2c and S2d illustrate the linear relationships between fluorescence intensities and [$O_2^{\bullet-}$] in a wide range from 10 nM to 30 μM, and the linear regression equation was F = 7679.81 [$O_2^{\bullet-}$] (μM)+116305.92 with a correlation coefficient of 0.998. Furthermore, the detection limit of 2.8 nM was calculated according to IUPAC based on triplicate measurements of the relative standard[1].

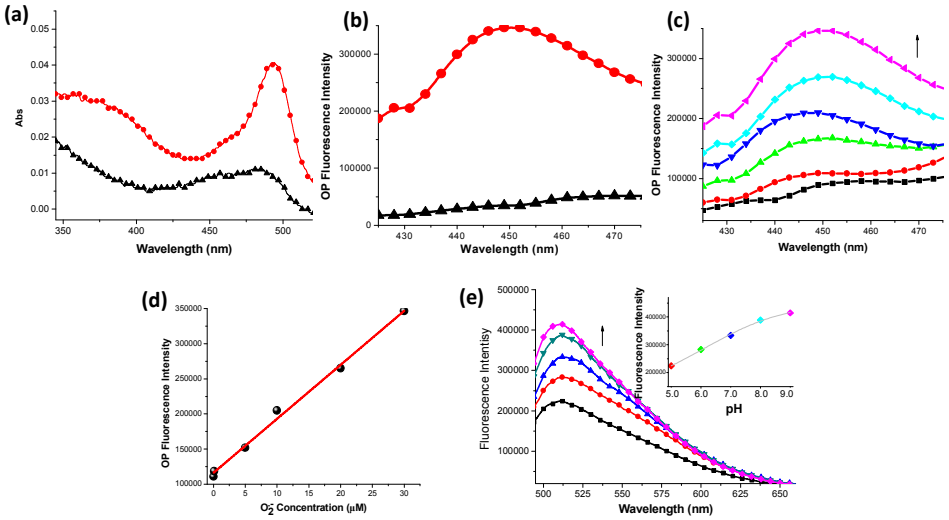

Figure S2. (a) The absorption spectra of CFT (10 μM) in PBS buffer (30 mM, pH 7.4, ▲) and after the addition of $O_2^{\bullet-}$ in PBS (30 mM, pH 9.0, ●). (b) OP emission spectra for CFT (10 μM) before (▲) and after (●) the addition of $O_2^{\bullet-}$ (30 μM). (c) One-photon fluorescence spectra of 10 μM CFT after adding $O_2^{\bullet-}$ of various concentrations (10 nM-30 μM). (d) A linear correlation between the fluorescence intensities and $O_2^{\bullet-}$concentrations. (e) Emission spectra of CFT (10 μM) at various pH values from 5.0 to 9.0. Insert: fluorescence intensities vs. pH with Ex/Em at 488/520.

5. The selectivity of CFT

To confirm the selectivity of CFT for $O_2^{\bullet-}$/pH, we examined the fluorescent responses in the presence of competing ROS and reactive nitrogen species (RNS) in PBS buffer. As shown in Fig.S3a and S3b, the blue fluorescence of CFT exhibited a high selectivity for $O_2^{\bullet-}$ and was unperturbed upon the presence of other ROS and RNS, as was the pH, indicating that the caffeoyl-based recognition site is highly selective for $O_2^{\bullet-}$. As displayed in Fig. S3c, under conditions of various pH, CFT produced coincident fluorescence changes in the presence of ROS and RNS, especially $O_2^{\bullet-}$, suggesting that the fluorescein-based recognition site has a high selectivity for pH. Generally, these results demonstrate that the caffeoyl-based and fluorescein-basedrecognition sites can exclusively respond to $O_2^{\bullet-}$or pH, respectively.

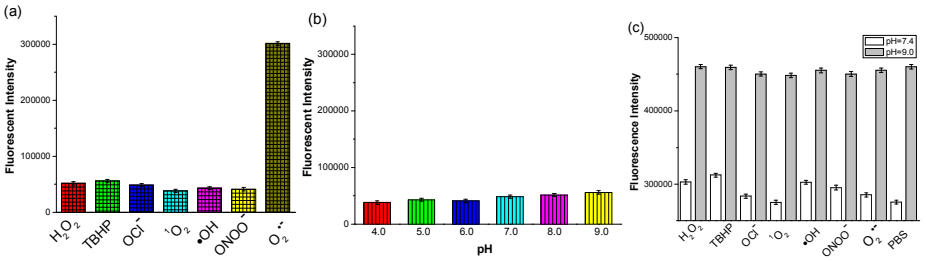

Figure S3. (a) Fluorescence responses of 10 μM CFT to various reactive oxygen and nitrogen species (20 mM H_2O_2, 200 μM TBHP, 200 μM NaClO, 200 μM $^1O_2$, 33 μM $ONOO^-$, 200 μM•OH and $O_2^{\bullet-}$ 20 μM). The bars represent the fluorescence intensities of PY-CA after the addition of each reactive species. All of the data were acquired in 30 mM PBS, pH 7.4, with λ_{ex} = 400 nm and λ_{em} = 450 nm. (b) Fluorescence responses at 10 μM CFT to various pH from 4.0 to 9.0, with λ_{ex} = 400 nm and λ_{em} = 450 nm. (c) Fluorescence responses of 10 μM CFT to various reactive oxygen and nitrogen species in 30 mM PBS, pH 7.4 or 9.0, with λ_{ex} = 488 nm and λ_{em} = 520 nm.

6. The fluorescence response time and reversibility of CFT

We next investigated the fluorescent response time and reversibility of CFT regarding $O_2^{\bullet-}$/pH. As shown in Fig. S4a, the addition of $O_2^{\bullet-}$ triggered instantaneous and significant fluorescence enhancement at 450 nm with 400 nm excitation. Similarly, the pH elevation immediately induced the fluorescence enhancement at 520 nm with 488 nm excitation (Fig. S4b). Indeed, the fast response is crucial for the real-time detection of $O_2^{\bullet-}$/pH. Moreover, the fluorescence was maintained nearly constant within 50 min, which indicated superior photostability. Furthermore, we inspected whether the fluorescent responses of CFT to $O_2^{\bullet-}$ and pH were reversible. As depictedin Fig.S4c and S4d, the fluorescence intensity dramatically increased by adding $O_2^{\bullet-}$ and was remarkably quenchedby treating with ascorbic acid (Vc). In the meantime, reducing the pH significantly decreased the CFT fluorescence, whereas increasing the pH recovered the fluorescence. A reversible cycle could be achieved at least three times with little fluorescence efficiency loss under the same conditions. Generally, CFT exhibited a reversible on-off-on type and instantaneous fluorescence responses toward alternate addition of $O_2^{\bullet-}$/Vc or acid/base medium.These results suggest that CFT can serve as a robust sensor to realize dynamic fluorescence imaging for $O_2^{\bullet-}$ and pH and affords an excellent opportunity for real-time examination of the cross-talk between these systems biological specimens.

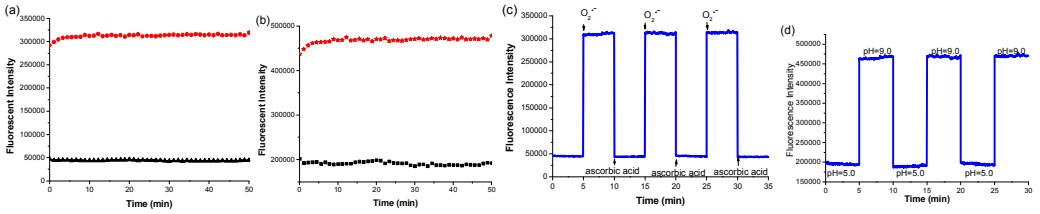

Figure S4. (a) The time course of one-photon fluorescence for 10 μM CFT before (▲) and after (●) the addition of $O_2^{\bullet-}$ with λ_{ex} = 400 nm and λ_{em} = 450 nm. (b) pH time course of one-photon fluorescence for 10 μM CFT in PBS buffer with pH=5.0 (■) and 9.0 (★), λ_{ex} = 488 nm and λ_{em} = 520 nm.. (c) 30 μM $O_2^{\bullet-}$ was added to CFT solution, and the solution was then treated with 2.0 mM Vc after 5 min. After 5 min, another 30 μM $O_2^{\bullet-}$ was added to the above mixture. The cycles were repeated three times. All of the one-photon spectra were acquired in 30 mM PBS buffer (pH 7.4) at λ_{ex} = 400 nm and λ_{em} = 450 nm. (d) pH fluorescent reversibility of CFT between pH 5.0 and 9.0 with λ_{ex} = 488 nm and λ_{em} = 520 nm.

7. Cytotoxicity assay

We assessed the potential toxicities of CFT against a 4T1 cell line. First, we seeded the 4T1 cells (10^6 cell mL^{-1}) into replicate 96-well microtiter plates at a total volume of 200 μL $well^{-1}$. After 12 h of culture as previously described, various probe concentrations (1×10^{-4}, 1×10^{-5}, 1×10^{-6} and 1×10^{-7} M) were added to the cells and incubated for 24 h. Subsequently, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg mL^{-1}, PBS) was added to each well. The MTT solution was removed after 4 h of incubation, and 150 μL of DMSO was added into each well. Finally, we measured the absorbance at 490 nm in a TRITURUS microplate reader. The results indicated that CFT has no marked cytotoxicity at concentrations below 5.1 mM.

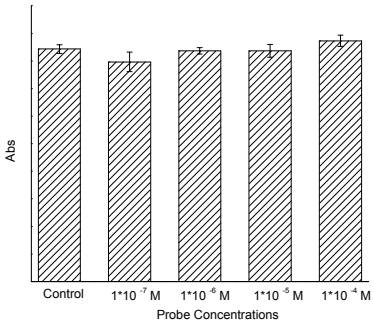

Figure S5. The MTT assay of CFT.

8. RNA interference and Western blot analysis

All of the siRNAs were purchased from GenePharma, and the siRNA sense strand sequences were from a previous work[2]. The siRNA sense strand sequences are as follows: 5'-AACGCAGAGCAGCGGAAAGAG-3'.

All the pre-treated cells were lysed in cell lysis buffer containing the protease inhibitor PMSF. The cell lysates were centrifuged at 15,000 rpm for 10 min at 4°C. All of the primary antibodies were from Abcam.And the following experiments were accomplished by Yuci Biotechnology Company. The Western blot analysis in Figure S6 showed that knock down of Drp1 enhanced the production of pro-apoptotic Bax and caspase-3 proteins while reducing the expression of anti-apoptotic Bcl-2.

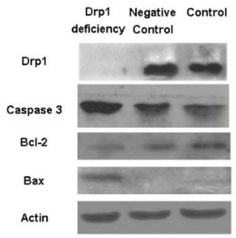

Figure S6. Expression of Drp1 and apoptosis-related proteins (caspase-3, Bcl-2 and Bax) was evaluated by immunoblot analysis in 4T1 cells that were co-transfected with Drp1 siRNA, control siRNA or nothing as a control.

9. Cell culture and Fluorescence imaging in living cells

4T1 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin at 37°C (w/v) in a 5% $CO_2$95% air incubator MCO-15AC

(SANYO, Tokyo, Japan). One day before imaging, the cells were detached and replanted onto glass-bottomed dishes.

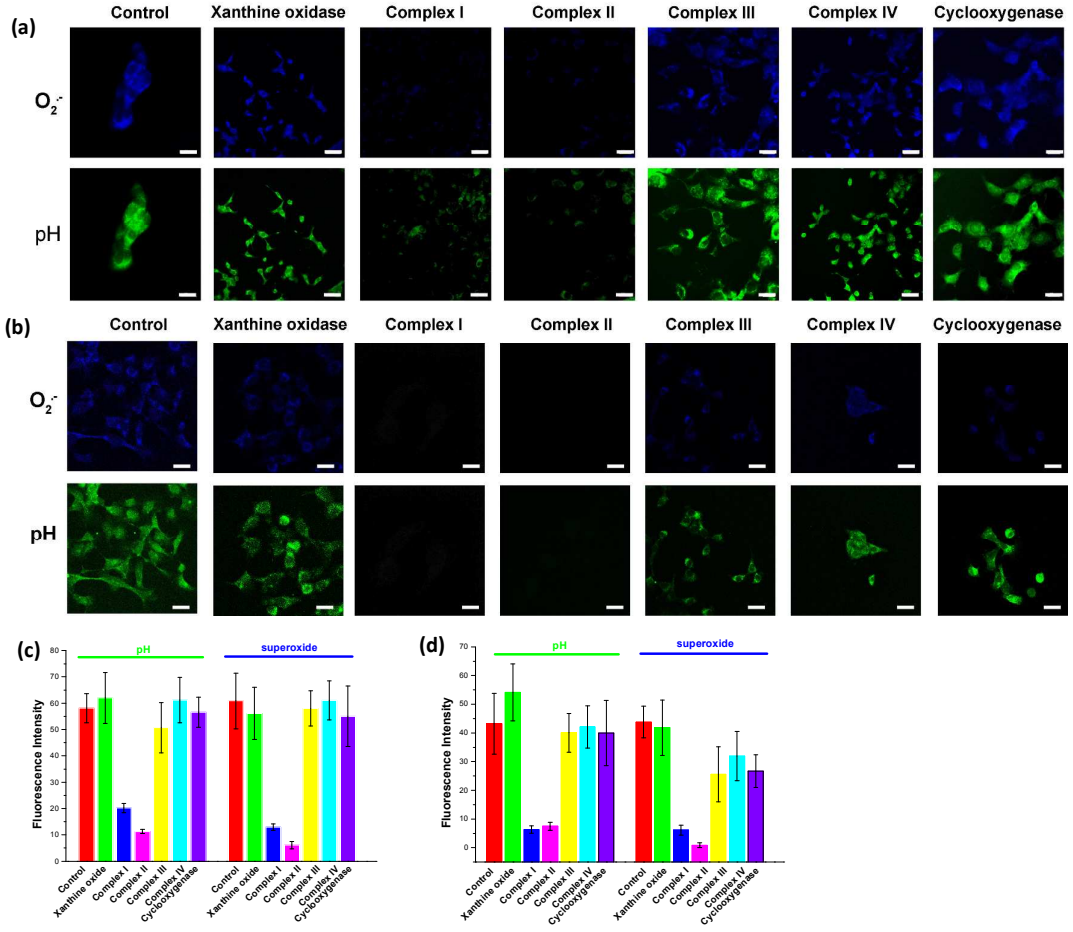

Figure S7. (a) Fluorescent images of Drp1 knockdown 4T1cells were pretreated with 10 μM CFT and different inhibitors for superoxide generation (10 μM rotenone for complex I, 100 μMmalonic acid for complex II, 100 μM 2,3-dimercaptopropanol for complex III, 1 mM KCN for complex IV, 100 purinol for xanthine oxidase and 200 μM Arcoxia for cyclooxygenase). (b) 4T1 cells pre-treated with 10 μM CFT and 100 μM mdivi-1. CFT-pretreated 4T1 cells were cultured with different inhibitors prior to the addition of mdivi-1 (100 μmol/L).10 μM rotenone for complex I, 100 μMmalonic acid for complex II, 100 μM 2,3-dimercaptopropanol for complex III, 1 mM KCN for complex IV, 100 purinol for xanthine oxidase and 200 μM Arcoxia for cyclooxygenase. (c and d) Relative fluorescence intensities of CFT-labeled 4T1 cells in (a and b). All of the fluorescence images from the blue channel (response to $O_2^{\bullet-}$) were excited at 405 nm, and the emission windows were collected at 430-480 nm. Fluorescence images from the green channel (response to pH) were excited at 488 nm, and the emission windows were collected at 500-550 nm. Scale bar = 20 μm. The cells shown are representative images from replicate experiments (n = 5).

10. Mouse models of melanoma

Eight- to ten-week-old Balb/c mice (female) were used for the tumor model. The tumor modelwas established by athoracic injection of4T1 cell suspensions (10^5 cellsmL^{-1}). When the tumor diameter reached 0.5 cm, the tumor-bearing animals were used for analysis. The permission of animal experiments was obtained from the Shandong normal university authorities.

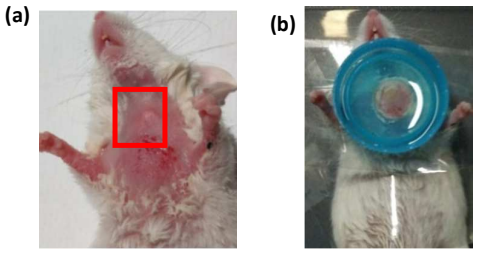

Figure S8. (a) Mouse tumor model. (b) Simple TP imaging equipment for water lenses in the tumor region.

REFERENCES

(1) Safaeian, M.; Solomon, D.; Castle, P. E. *Spectroscopy* **2003**, *18*, 112.