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Supplementary Information

Elucidating the Relationship between Superoxide Anion Levels and Lifespan Using an Enhanced Two-photon Fluorescence Imaging Probe

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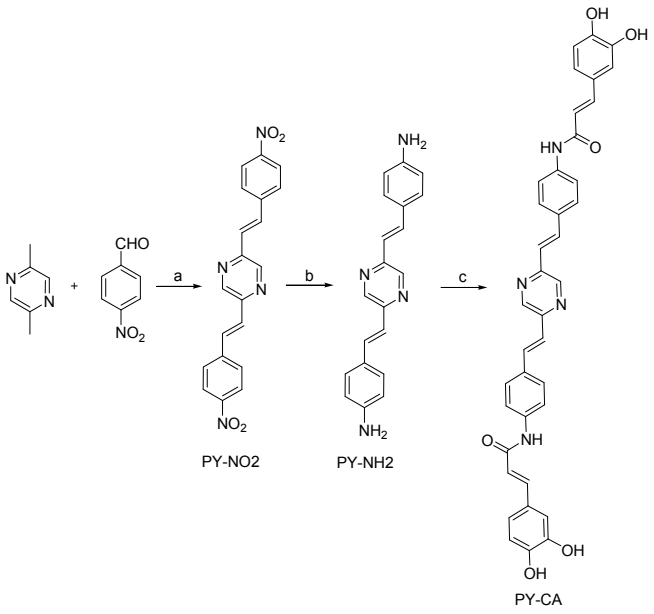
Materials and reagents

All chemicals were available commercially and the solvents were purified by conventional methods before use. Caffeic acid was from Adamas Reagent. Reactive oxygen species were as follows. H_2O_2, tert-butylhydroperoxide (TBHP), and hypochlorite (NaOCl) were delivered from 30%, 70%, and 10% aqueous solutions respectively. Hydroxyl radical (•OH) was generated by reaction of 1 mM Fe^{2+} with 200 μM H_2O_2. Nitric oxide (NO) was used from stock solution prepared by sodium nitroprusside. Singlet oxygen ($^1O_2$) was prepared by the ClO^-/H_2O_2 system. Peroxynitrite was used from stock solution 10 mM in 0.3 M NaOH. Superoxide ($O_2^{\cdot-}$) was delivered from KO_2 in DMSO solution.

^{1}HNMR spectra were determined by 400 MHz using Bruker NMR spectrometers. The mass spectra were obtained by Bruker maXis ultra-high resolution-TOF MS system. All OP fluorescence measurements were carried out at room temperature on FLS-920 Edinburgh fluorescence spectrometer. Two-photon excited fluorescence spectra were measured using a Tsunami 3941-M3-BB: Ti: sapphire femtosecond laser as exciting light source (800 nm) with a pulse width of <150 fs and a repetition rate of 80 MHz, and USB2000 (bought from Ocean Optics Inc.) was used as the recorder.

One-photon fluorescent images were acquired on a Leica TCS SP5 confocal laser-scanning microscope with an objective lens (×10 and ×20). The excitation wavelength was 405 nm (5 mW). Following incubation, the cells were washed three times with DMEM without FBS and imaged. The TP images were acquired with Leica TCS MP5 (25× water objective) and Leica TCS SP8 (20× water objective). All the Ti:sapphire laser was used to excite the specimen at 800 nm and transmissivity was 3%. The fluorescence collection window was 500-550 nm.

Synthesis of probe PY-CA



Scheme S1. Synthesis of PY-CA; a) phthalic anhydride, 180℃; b) sodium polysulfide, ethanol, 78℃; c) EDC, HOBT, DMF, DCM, r.t..

2,5-di(4'-nitrostyrene)pyrazine (PY-NO_2) and 2,5-di(4'- aminostyrene)pyrazine (PY-NH2) were prepared by modified methods reported in the literature. Synthesis of PY-CA is described below. Triethylamine (1.0 mL), 1-hydroxybenzotriazole (0.636 g, 3.0 mmol) and EDC (0.576 g, 3.0 mmol) were added to a solution containing caffeic acid (0.525 g, 3.0 mmol) and PY-NH2 (0.314 g, 1.0 mmol) in dinethylformamide (1.2 mL) and dichloromethane (8.0 mL). After the mixture was stirred over night at room temperature under nitrogen, it was concentrated under vacuum. The residue was purified by preparative thinlayer chromatography of silica gel GF_{254} with toluene/ methyl alcohol/ ethyl acetate/ (4/ 0.5/ 2) as eluent and light yellow product was obtained.

PY-NH2. ^{1}H NMR (400 MHz, DMSO): δ 8.55 (s, 4H), δ 8.43 (s, 2H), δ 7.56 (d, J=16.0, 2H), δ 7.35 (d, J=8.0, 4H), δ 6.99 (d, J=16.0, 2H), δ 6.59 (d, J=8.0, 4H); ^{13}C HNMR (400 MHz, DMSO): δ 150.27, 144.11, 142.27, 134.29, 129.08, 129.01, 118.95, 114.31; MS data, m/z calcd for [$C_{20}H_{18}N_4$+H] 315.1565, found 315.1600.

PY-CA. ^{1}H NMR (400 MHz, DMSO): δ 8.30 (s, 2H), δ 8.16 (d, J=8.0, 4H), δ 7.92 (d, J=8.0, 4H), δ 7.9278 (d, J=16.0, 2H), δ 7.63 (d, J=8.0, 4H), δ 7.47 (d, J=16.0, 2H), δ 7.45 (d, J=16.0, 2H), δ 7.43 (s, 4H), δ 7.37 (d, J=16.0, 2H), δ 7.35 (s, 4H); ^{13}C HNMR (400 MHz, DMSO): δ 164.38, 162.96, 143.24, 132.48, 132.03, 129.58, 129.13, 128.26, 127.94, 125.96, 124.34, 120.20, 110.08, 113.40, 110.72, 110.19, 106.87; MS data, m/z calcd for [$C_{38}H_{30}N_4O_6$+H] 639.2165, found 639.2265.

The compound PY-CAO can be obtained via oxidation reaction between PY-CA and $O_2^{\cdot-}$. After adding excessive $O_2^{\cdot-}$, dark yellow product was obtained. 1H NMR (400 MHz, DMSO): δ 8.71 (s, 2H), δ 8.31 (d, J=16.0, 2H), δ 8.04 (d, J=8.0, 4H), δ 7.92 (d, J=16.0, 2H), δ 7.86 (d, J=16.0, 2H), δ 7.70 (d, J=8.0, 4H), δ 7.44 (d, J=16.0, 2H), δ 7.21 (s, 2H), δ 7.08 (d, J=16.0, 2H), δ 6.94 (d, J=16.0, 2H); ^{13}C HNMR (400 MHz, DMSO): δ 178.28, 174.78, 169.01, 166.52, 160.78, 157.46, 149.92, 148.84, 148.74, 143.96, 130.06, 128.81, 124.57, 124.35, 117.39; MS data, m/z calcd for [$C_{38}H_{26}N_4O_6$+H] 635.1852, found 635.2353.

The selectivity of PY-CA

The selectivity of PY-CA was studied (Figure S1). It is showed that PY-CA was unperturbed upon the addition of various concentrations of other reactive species.

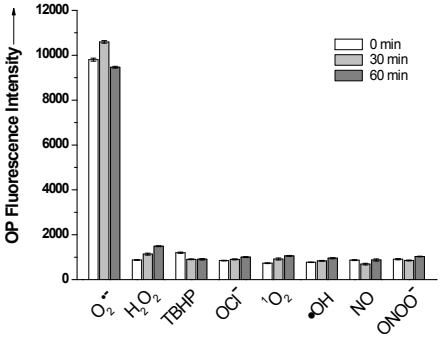


Fig. S1 Fluorescence responses of 10 μM PY-CA to various reactive oxygen and nitrogen species (20 mM H_2O_2, 200 μM TBHP, 200 μM NaClO, 200 μM $^1O_2$, 33 μM $ONOO^-$, 200 μM ·OH, 200 μM NO and $O_2^{\cdot-}$ 20 μM). Bars represent fluorescence intensity of PY-CA at 0, 30 and 60 min after the addition of each reactive species. All of the data were acquired in 30 mM Tris, pH 7.4, with λ_{ex} = 400 nm.

Instantaneous response of PY-CA

In Figure S2, PY-CA displayed rapid response and good photostability. The instantaneous response implied

the advantage of PY-CA for dynamic determine $O_2^{\cdot-}$ in cells and in vivo.

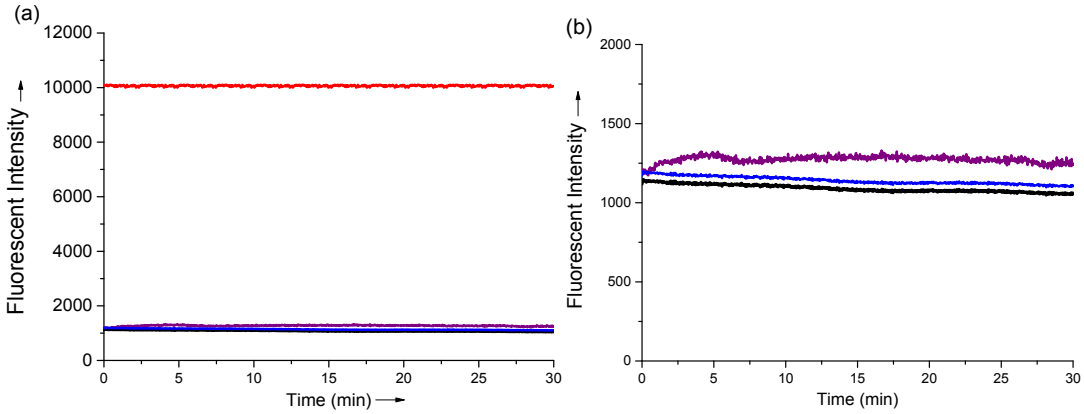


Fig. S2 The time course of one-photon fluorescence. Black line: 10 μM PY-CA. Red line: 10 μM PY-CA and 20 μM $O_2^{\cdot-}$. Blue line: adding 2.0 mM GSH to the reaction system containing10 μM PY-CA and 20 μM $O_2^{\cdot-}$. Violet line: adding 1.0 mM ascorbic acid to the reaction system containing10 μM PY-CA and 20 μM $O_2^{\cdot-}$. Data were acquired in 30 mM Tris, pH 7.4, with λ_{ex} = 400 nm. Fig.S2 was the magnification for the onset region of a.

Cytotoxicity assay

HepG2 cells (10^5 cell mL^{-1}) were seeded at a density of 1×10^6 cells/mL in replicate 96-well microtiter plates to a total volume of 200 μL $well^{-1}$. Plates were maintained at 37 °C in a 5% CO_2/95% air incubator for 12 h. The cells were incubated for an additional 24 h with different concentrations probe (1×10^{-3}, 1×10^{-4}, 1×10^{-5} 1×10^{-6} and 1×10^{-7} M). Subsequently, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg mL^{-1}, PBS) was then added to each well. After 4 h incubation, the remaining MTT solution was removed, and 150 μL of DMSO was added into each well, followed by further incubation for 4 h at 37 °C. Absorbance was measured at 490 nm in a TRITURUS microplate reader (Figure S3). Data indicated that PY-CA has no marked cytotoxicity at concentrations below 1.31 mM.

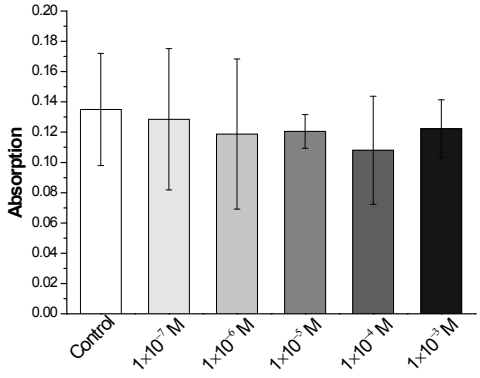


Fig. S3 MTT assay. The cells were incubated with PY-CA of different concentrate for 24 h. IC_{50} values was 1.31 mM.

The reversibility of PY-CA

The reversibility of the probe was tested (Figure S4). This reversible cycle can be repeated for three times

more under the same conditions. The reversibility implied the advantage of PY-CA for dynamic determine $O_2^{\cdot-}$ in cells and in vivo.

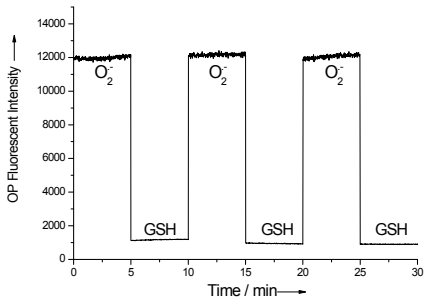


Fig. S4 The reversibility of PY-CA. 20 μM $O_2^{\cdot-}$ was added into PY-CA solution, after 5 min, the solution was treated with 2.0 mM GSH. When the fluorescence returned to the baseline level, another 20 μM $O_2^{\cdot-}$ was added to the above mixture after 5 min. The cycles were repeated three times. All of the one-photon spectra were acquired in 30 mM Tris buffer (pH 7.4) at λ_{ex} = 400 nm.

Cell culture and mouse models of melanoma

HepG2 cells and 4T1 cells were cultured in high glucose DMEM (4.5 g of glucose/L) 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 were replanted on glass-bottomed dishes.

B16 melanoma cells were maintained in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum and penicillin/streptomycin. Eight- to ten-week-old C57BL/6 mice (male) were used for the tumor models. Mice received a subcutaneous injection of various doses of B16 cells (10^5 cells). When tumor diameter reached 2 cm, small animal tumor models can be obtained. The C57BL/6 mice were obtained from Shandong University Laboratory Animal Center. The experiments were approved by the institutional committee. All the animal experiments were carried out in accordance with the relevant laws and guidelines issued by the Ethical Committee of Shandong University.

One-photon fluorescent imaging

We employed PY-CA to monitor $O_2^{\cdot-}$ fluctuations in HepG2 cells simulated with 2-methoxyestradiol (2-ME). PY-CA (10 μM) pre-incubating HepG2 cells showed strong fluorescence upon stimulation of 1.0 μg/mL 2-ME (Fig. S5b) comparing to the control without treatment of 2-ME (Fig. S5a), indicating the rise of $O_2^{\cdot-}$ concentrations. After adding 1.0 mM ascorbic acid to these cells, fluorescence decreased immediately (Fig. S5c). One-photon imaging indacated that PY-CA can dynamically and selectively visualize intracellular $O_2^{\cdot-}$ level changes

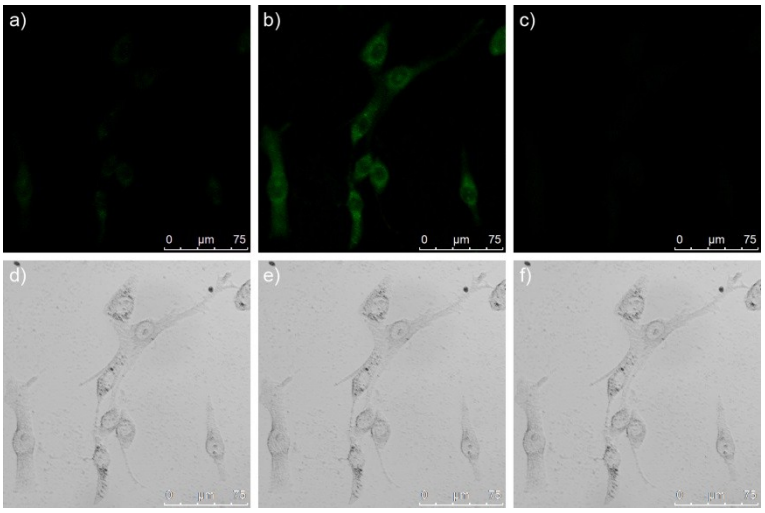


Figure S5. In situ OP fluorescent imaging of $O_2^{\cdot-}$ in HepG2 cells. OP images were captured after addition of 10 µM PY-CA in a same one confocal dish (a-e). (a) Cells only incubated with 10 µM PY-CA for 10 min at room temperature. (b) Cells pre-incubated with 10 µM PY-CA were treated with 2-ME (1.0 µg/mL) for 30 min. (c) the 2-ME-stimulated cells were incubated with 1.0 mM ascorbic acid for 30 min. (d-f) Bright-field image of figs S5a-S5c. One-photon excitation wavelength was at 405 nm and emission windows: 500-550 nm. Scale bar = 75 µm.

Lifetime analysis

C. elegans were cultured at 20 °C on solid nematode growth media (NGM) with Escherichia coli strain OP50. Eggs were placed on the plates (100-200 eggs per plate), and left to hatch and grow to the beginning of the adult stage. All compounds were added into the NGM media every two days and worms were moved to new assay plates every 2 days. To assess the survival rate, the worms were prodded with a platinum wire every day, and those that failed to respond were scored as dead.

To analyze $O_2^{\cdot-}$ concentrations in different life stages of *C. elegans*, PY-CA was applied in fluorescence imaging in vivo (Figure S6). Wild-type worms was stimulated with paraquat (4 mM) to raise $O_2^{\cdot-}$ concentrations, meanwhile, 10 µM Tiron was used to depress $O_2^{\cdot-}$ concentrations. Comparing with control, stronger fluorescence was observed in the worms treated with paraquat, and weaker fluorescence in the worms treated with Tiron. Our approach provides direct visualization evidence how the $O_2^{\cdot-}$ levels affect *C. elegans* lifespan.

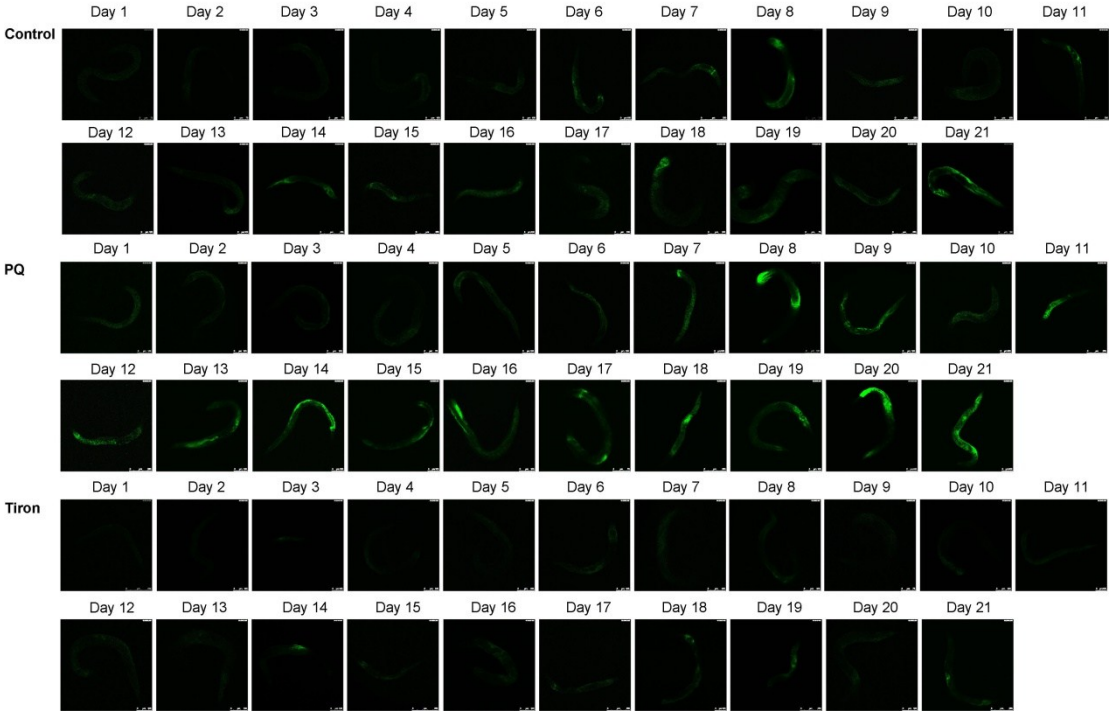


Figure S6 OP fluorescent images of $O_2^{\cdot-}$ in wild-type *Caenorhabditis elegans (C. elegans)* on various adult days. The worms pre-treated with water (Control), paraquat (PQ) and Tiron were incubated with 10 μM PY-CA. Graphs show $O_2^{\cdot-}$ fluctuations of wild-type *C. elegans* in lifespan of 21 days.

Measurement of OP fluorescence quantum yields and two-photon cross section

OP fluorescence quantum yields were calculated using the relative fluorescence method byusing Coumarin 307 as the reference dye.[1] The two-photon absorption cross section (δ) was measured by using femto second fluorescence method. PY-CAO were dissolved in 30 mM Tris buffer at the concentration of 10 μM and then the two-photon induced fluorescence intensity was measured at 800 nm by using Rhodamine 6G in MeOH as the reference.[2] The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using $\delta=\delta_r (F_s \Phi_r n_r C_r)/(F_r \Phi_s n_s C_s)$, where the subscripts s and r refer to the sample and the reference material, respectively. δ is the TPA cross sectional value, C is the concentration of the solution, n is there refractive index of the solution, F is two-photon excited fluorescence integral intensity and Φ is the fluorescence quantum yield.

REFERENCES

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