Electronic Supplementary Information

A novel fluorescent sensor for detection of highly reactive oxygen species, and for imaging such endogenous hROS in the mitochondria of living cells

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Dye synthesis

The synthetic route for PTZ-Cy2 is outlined in Scheme S1.

Scheme S1. Synthetic route of dye PTZ-Cy2. i) DMF, 1,2-dichloroethane, POCl₃, reflux 8 h, yield 63%; ii) toluene, CH₃CH₂I, reflux 10 h, yield 92%; iii) ethanol, piperidine, reflux 12 h, yield 65%.

Intermediate products a and b were synthesized according to literature procedures. ¹⁻²

Synthesis of 3-(2-(1’,3’,3’-trimethyl-indolium-2’-yl) vinyl]-phenothiazine (PTZ-Cy2)

1-Ethyl-2, 3, 3’-trimethylindolenium quaternized salt b (1.58 g, 5.0 mmol), and aldehyde a (2.4 g, 4.5 mmol) were added to a 100 mL flask with 50 mL ethanol, followed by catalytic piperidine (1.0 mL). The resulting mixture was stirred for 12 h under reflux. The residue was recrystallized from ethanol to give the desired product in 65% yield. 1H NMR (400 MHz, d₆-DMSO) δ 9.55 (s, 1H), 8.21 (d, J = 16.0 Hz, 1H), 7.84 (m, 4H), 7.57 (m, 2H), 7.36 (d, J = 16.0 Hz, 1H), 7.03 (s, J = 7.2 Hz, 1H), 6.95 (d, J = 7.2 Hz, 1H), 6.84 (s, J = 7.2 Hz, 1H), 6.72 (m, 2H), 4.61 (d, J = 7.2 Hz, 2H), 1.75 (s, 6H), 1.41 (t, J = 7.2 Hz, 3H). HRMS-ESI: m/z calcd. M⁺ for C₂₆H₂₅N₂S⁺, 397.1733; found, 397.1729.
$^{13}$C NMR (100 MHz, d$_6$-DMSO) δ 180.55, 153.29, 146.91, 143.90, 140.99, 138.95, 129.86, 129.46, 128.99, 128.68, 128.42, 127.98, 123.99, 123.46, 122.29, 114.77, 114.74, 108.27, 105.58, 73.98, 52.03, 30.16, 26.41, 14.00, 10.88.

3-[2-(1',3',3'-thimethyl-indolium -2'-yl)vinyl]-5-oxo-phenothiazine (OPTZ-Cy2)

$^1$H NMR (400 MHz, d$_6$-DMSO) δ 11.67 (s, 1H), 8.97 (s, 1H), 8.55 (m, 2H), 8.04 (d, $J = 7.6$ Hz, 1H), 7.90 (d, 2H), 7.72 (d, 1H), 7.63 (m, 3H), 7.52 (m, 2H), 7.35 (t, $J = 7.2$ Hz, 1H), 4.72 (q, $J = 7.2$ Hz, 2H), 1.83 (s, 6H), 1.48 (t, $J = 7.2$ Hz, 3H). HRMS-ESI: m/z calcd. M$^+$ for C$_{26}$H$_{30}$N$_2$O$^+$, 413.1682; found, 413.1681.

3-carbaldehyde 5-oxo-phenothiazine (OPTA)

$^1$H NMR (400 MHz, d$_6$-DMSO) δ 11.56 (s, 1H), 9.96 (s, 1H), 8.55 (s, 1H), 8.05 (m, 2H), 7.70 (m, 1H), 7.51 (m, 2H), 7.33 (t, $J = 7.2$ Hz, 1H). HRMS-ESI: m/z calcd. M for C$_{13}$H$_9$NO$_2$S, 243.0354; found, 243.0358.

Preparation of stock solutions for generation of ROS$^{3-6}$

(a) H$_2$O$_2$

H$_2$O$_2$ was diluted appropriately in water. The concentration of H$_2$O$_2$ was determined based on the molar extinction coefficient at 240 nm (43.6 M$^{-1}$ cm$^{-1}$). Then, a H$_2$O$_2$ stock solution in water was prepared.

(b) •OH

To a solution of H$_2$O$_2$ in 100 μM sodium phosphate buffer at pH 7.4 as a cosolvent, the FeSO$_4$ solution (10 μM) was added at room temperature. Then, •OH was generated from Fe$^{2+}$ and H$_2$O$_2$ (Fenton reaction).

(c) OCl$^-$

NaOCl solution was diluted appropriately in 0.1 M NaOH aq. The concentration of OCl$^-$ was determined based on the molar extinction coefficient at 292 nm (350 M$^{-1}$ cm$^{-1}$). Then, a OCl$^-$ stock solution in 0.1 M NaOH aq. was prepared.

(d) Generation of •O$_2^-$

Superoxide (•O$_2^-$) was added as solid KO$_2$.

(e) $^1$O$_2$

A solution of NaMoO$_4$ was added to a solution of H$_2$O$_2$ in 0.1 M sodium phosphate buffer at pH 7.4 as a cosolvent at room temperature.

Determination of the detection limit

The detection limit was calculated based on the method used in the previous literature$^7$. The fluorescence emission spectrum of PTZ-Cy2 was measured by three times and the standard deviation of blank measurement was achieved. The fluorescence intensity at 595 nm was plotted as a concentration of NaClO. The detection limit was calculated with the following equation:
Detection limit = $3\sigma/k$ \hspace{1cm} (1)

Where $\sigma$ is the standard deviation of blank measurement, $k$ is the slope between the fluorescence intensity versus hROS concentration.

**Live cell incubation**

HeLa cells were cultured in DMEM (Invitrogen) supplemented with 10% FCS (Invitrogen). One day before imaging cells were seeded into 24-well flatbottomed plates. The next day, the cells were incubated with 8.0 μM dye for 40 min at 37 °C under 5% CO₂ and washed with phosphate-buffered saline (PBS) three times.

Hela cells pre-treated with PMA (2 ng mL⁻¹) for 40 min and then incubated with PTZ-Cy2 (8 μM) plus MitoTracker Deep Red FM (1 μM) for 30 min at 37 °C. The cells were washed with PBS buffer and the fluorescence images were acquired.

**Fluorescence imaging**

Fluorescence imaging in cells were obtained with spectral confocal multiphoton microscopes (Olympus FV1000 confocal laser scanning microscope).

**Photostability**

PTZ-Cy2, OPTZ-Cy2, OPTA were dissolved in DMSO-water (5:5 v) at a concentration of 10.0 μM, respectively. The solutions were irradiated under a 500 W iodine tungsten lamp for 2 h at a distance of 250 mm away. An aqueous solution of sodium nitrite (50.0 g/L) was placed between the samples and the lamp as a heat filter. The photostabilities were expressed in the terms of remaining absorption (%) calculated from the changes of absorbance at the absorption maximum before and after irradiation by iodine tungsten lamp.
Table S1 Spectral data of dyes.

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<th>Dyes</th>
<th>Solvents</th>
<th>λ&lt;sub&gt;abs&lt;/sub&gt; (nm)</th>
<th>λ&lt;sub&gt;em&lt;/sub&gt; (nm)</th>
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**Figure S1.** The absorption (left) and emission (right) spectra of dye PTZ-Cy2 in water. λ<sub>ex</sub> = 345 nm.

**Figure S2.** The absorption (left) and emission (right) spectra of dye OPTZ-Cy2 in water. λ<sub>ex</sub> = 405 nm.
**Figure S3.** The absorption (left) and emission (right) spectra of dye OPTA in water. $\lambda_{ex} = 405$ nm.

**Figure S4** a) Changes in the fluorescence spectrum of PTZ-Cy2 (10 μM) upon addition of NaClO (0-60 μM). Each spectrum was recorded after 5 min in water. b) Changes in the fluorescence spectrum of PTZ-Cy2 (10 μM) upon addition of $\cdot$OH (0-30μM). Each spectrum was recorded after 3 min in water. $\lambda_{ex} = 340$ nm.

**Figure S5** The fluorescence changes at 595 nm, upon addition of lower concentrations of NaClO at 1–10 μM. Excitation: 450 nm.
**Figure S6**  
a) Plot of the absorption intensity ratios at 470 nm and 550 nm of PTZ-Cy2 (10 μM) upon addition of NaClO (0–30 μM). $\lambda_{ex} = 340$ nm. b) Fluorescence intensity at 470 nm of PTZ-Cy2 (10 μM) upon addition of NaClO (0–30 μM). Conditions: each spectrum was recorded after 5 min in water. $\lambda_{ex} = 450$ nm.

**Figure S7**  
a) Plot of the absorption intensity ratios at 470 nm and 550 nm of PTZ-Cy2 (10 μM) upon addition of •OH (0–10 μM). b) Fluorescence intensity at 470 nm of PTZ-Cy2 (10 μM) upon addition of •OH (0–12 μM). $\lambda_{ex} = 340$ nm. Conditions: each spectrum was recorded after 3 min in water. $\lambda_{ex} = 450$ nm.

**Figure S8.**  
a) Time dependent fluorescence intensity changes of PTZ-Cy2 (10 μM) at 595 nm in the presence of 50 equiv •OH in water (180min). b) Time dependent fluorescence intensity changes of PTZ-Cy2 (5 μM) at 595 nm in the presence of 20 equiv NaClO in water (200min), $\lambda_{ex} = 340$ nm.
Figure S9. a) Changes in the fluorescence emission spectrum of PTZ-Cy2 (10 μM) with increases of NaClO concentration (0-30 μM) at 595 nm in water. b) Changes in the fluorescence emission spectrum of PTZ-Cy2 (10 μM) with increases of the •OH concentration (0-10 μM) at 595 nm in water, \( \lambda_{ex} = 450 \) nm.

Figure S10. Influence of pH on fluorescence for PTZ-Cy2 (20 μM) over the range pH 3–8. \( \lambda_{ex} = 340 \) nm.

Figure S11. a) Photo-fading of dyes (PTZ-Cy2, OPTZ-Cy2, OPTA) in solvent mixture with the ratio of DMSO-water 5:5 v/v with radiation by a 500 W iodine-tungsten lamp. PTZ-Cy2: \( \lambda_{abs} = 550 \) nm, OPTZ-Cy2: \( \lambda_{abs} = 470 \) nm, OPTA: \( \lambda_{abs} = 350 \) nm. b) Time-profile of the emission intensities of compound PTE-Cy in water at room temperature for 72 h. The fluorescent data were collected at 595 nm.
**Figure S12.** PTZ-Cy2 (20 μM) was loaded into HeLa cells for 30 min. a) Green emission of the PTZ-Cy2 (470 ± 20) nm; b) red emission of the PTZ-Cy2 (590 ± 20) nm, c) the green-red merged image with bright-field image. \( \lambda_{ex} = 405 \) nm.

**Figure S13.** PTZ-Cy2 (20 μM) was loaded into HeLa cells for 1.5 hours. a) Green emission of the PTZ-Cy2 (470 ± 20) nm; b) red emission of the PTZ-Cy2 (590 ± 20) nm, c) the green-red merged image with bright-field image. \( \lambda_{ex} = 405 \) nm.

**Figure S14.** Hela cells pre-treated with NaClO (100 μM) for 40 min and then incubated with PTZ-Cy2 (8 μM) for 30 min. a) Green emission of the PTZ-Cy2 (470 ± 20) nm. b) Red emission of the PTZ-Cy2 (590 ± 20) nm. (c) The green-red merged image with bright-field image. \( \lambda_{ex} = 405 \) nm.
**Figure S15.** Hela cells pre-treated with PTZ-Cy2 (8 μM) for 30 min and then incubated with NaClO (100 μM) for 30 min. (a) Green emission of the PTZ-Cy2 (470 ± 20) nm. (b) Red emission of the PTZ-Cy2 (590 ± 20) nm. (c) The green-red merged image with bright-field image. λ_exc = 405 nm.

**Figure S16.** HPLC chromatograms of probe PTZ-Cy2, a) after reaction with NaOCl for 10 min, b) after reaction with •OH for 10 min.

*In vitro* testing: two fluorescent substances, OPTZ-Cy2 and OPTA, were generated when NaClO and were added slowly to the PTZ-Cy2 solution. To confirm the formation of these substances, the partial MS spectra of the reaction of PTZ-Cy2 with NaClO and •OH are shown in Fig S11.
Figure S17. MS monitoring oxidation of the hROS with PTZ-Cy2 process.

References

The $^1$H-NMR and $^{13}$C-NMR spectra of the dyes
**Figure S18.** The $^1$H-NMR and $^{13}$C-NMR spectra of PTZ-Cy2 in DMSO.

**Figure S19.** The $^1$H-NMR spectra of OPTZ-Cy2 in DMSO.
Figure S20. The $^1$H-NMR spectra of OPTA in DMSO.