Supporting information

A NIR Fluorescent Probe: Imaging Endogenous Hydrogen Peroxide During Autophagy Process Induced by Rapamycin

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Materials and methods
All solvents and reagents used were reagent grade and were used without further purification unless otherwise stated. Rhodamine 123 was purchased from Life Technologies Co. (USA). Flash column chromatography was performed using silica gel (100 - 200 mesh) obtained from Qingdao Ocean Chemicals. Mass spectrometric data were achieved with HP1100LC/MSD MS and an LC/Q-TOF-MS instruments. $^1$H NMR and $^{13}$C NMR spectra were recorded on a VARIAN INOVA-400 (or a Bruker Avance II 400 MHz or 500 MHz) spectrometer. Chemical shifts (δ) were reported as ppm in CDCl$_3$ with TMS as the internal standard. The solution of Cy-B was dissolved in dimethyl sulfoxide(DMSO) at a concentration of 1mM as the stock solution and stored in a refrigerator for use. Fluorescence spectra were performed on a VARIAN CARY Eclipse fluorescence spectrophotometer (Serial No. FL0812-M018). Excitation and emission slit widths were modified to adjust the fluorescence intensity to a suitable range. Absorption spectra were measured on a Perkin Elmer Lambda 35 UV/VIS spectrophotometer (Perkin Elmer). All pH measurements were performed using a Model PHS-3C meter. Slight pH variations in the solutions were achieved by adding the minimum volumes of HCl or NaOH (1 M). The fluorescence quantum yields were determined with Absolute PL Quantum Yield Spectrometer. Stock solutions or generation of ROS were prepared according to our previous report$^1$. Each experiment was carried out in three replicates (n = 3).

Photosatbility test
The photostability test was carried out in square cross-section quartz cells (1 $\times$ 1 cm) and solutions of the samples were irradiated with a 500 W iodine-tungsten lamp at room temperature. To eliminate the heat and absorb short wavelength light, a cold trap (3 L solution of 50 g/L NaNO$_2$ in 10 cm (width) $\times$ 30 cm (length) $\times$ 20 cm (height) was set up between the cells and the lamp. The distance between the lamp and the cold trap was 20 cm while the distance between the cold trap and the cells was 30 cm. The irreversible bleaching of the dyes at the absorption peak was monitored as a function of time. Samples were tightly sealed, but not deoxygenated with nitrogen before the test.

Determination of the detection limit
The detection limit was calculation based on the fluorescence titration (Figure 2a) of Cy-B in the presence of H$_2$O$_2$ (0-18μM) by using the following equation:
Detection limit = $3\sigma/k$
Where $\sigma$ is the standard deviation of the blank measurement, k is the slope of the fluorescence intensity at 709 nm versus H$_2$O$_2$ concentration.
**Living Cell imaging experiments**

MCF-7 cells and HL-7702 cells were purchased from Institute of Basic Medical Sciences (IBMS) of the Chinese Academy of Medical Sciences and cultured in DMEM medium containing 10% FBS and 1% antibiotics (penicillin/streptomycin, 100 U/mL) at an atmosphere of 5%/95% CO$_2$/air at 37 °C. As for imaging, the cells were seeded in 24-well flat-bottomed plates and then incubated for 24 h at 37 °C under 5% CO$_2$. Before imaging, cells are stained with Cy-B for 15 min and then washed with PBS (phosphate-buffered saline) for three times. In regard to exogenous detection, cells were then treated with 100 μM H$_2$O$_2$ for 60 min; As for the endogenous study, cells were then stimulated with 250 nM rapamycin for 90 min. Finally, fluorescence imaging was performed using an OLYMPUSFV-1000 inverted fluorescence microscope with a 60 × objective lens. Under the confocal fluorescence microscope, Cy-B was excited at 635 nm and emission was collected at 700-750 nm.

**MTT assay**

The cytotoxicity was evaluated by an MTT assay. MCF-7 cells were prepared in a 96-well plate with a concentration of 1 × 10$^5$ cells/mL in 100 μL DMEM medium supplemented with 10% FBS. After 24 h attachment at an atmosphere containing 5% CO$_2$, the cells were then treated with 2, 4, 6, 8, 10, 20 μM Cy-B in 100 μL medium for 48 h. Cells cultured without Cy-B is used as control. Each group has six replicates to eliminate contingency. After that, the medium was removed and 100 μL (5 mg/ml in PBS) MTT tetrazolium solution was added to every well and cultured for another 4 h at the same atmosphere. Finally, the MTT tetrazolium solution was removed carefully and 100 μL dimethyl sulfoxide (DMSO) was then added to resolve the formed formazan crystal. The plate was then shaken for 10 min and the absorbance was determined on a microplate reader (Thermo Fisher Scientific) at 570 nm and 630 nm. The viability was expressed as a percent of the controlled one using the following equation:

$$\text{Cells viability} (\%) = \frac{(\text{OD}_{\text{dye}} - \text{ODK}_{\text{dye}})}{(\text{OD}_{\text{control}} - \text{ODK}_{\text{control}})} \times 100$$

**Fluorescence imaging in mice**

All procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources and the National Research Council, and were approved by the Institutional Animal Care and Use Committee of the NIH. In this study, healthy mice (seven weeks old, 20-25 g) were used and the abdominal fur was removed. The mice were anesthetized and then imaged through the NightOWL II LB983 small animal in vivo imaging system equipped with a sensitive Charge Coupled Device (CCD) camera, with the excitation at 630 nm and the 700 nm emission filter.

**Synthesis of compounds**
Scheme S1. Synthetic procedures of Cy-B and its intermediates

Synthesis of 1
1 was synthesized by the reported methods.²

Synthesis of Cy-OH
Cy-OH was synthesized by the reported methods.³

Synthesis of 2
200 mg Cy-OH (0.50 mmol) was dissolved in 6 ml dichloromethane and triethylamine mixed solution (v/v = 1:1) at 0 °C. Then, 423 mg triflic anhydride (1.50 mmol) was added dropwise under continuously stirring. After adding all the triflic anhydride, the solution was stirred for another 30 min at 0 °C and then heat up to room temperature for 1 h. Finally, the reaction mixture was washed by 20 ml water and extracted with 30 ml dichloromethane for 3 times. The organic phase was evaporate to dryness and the crude product was purified by silica gel column chromatography with CH₂Cl₂/CH₃OH (40:1) and get the purple solid (182 mg, 68.7%).¹H NMR (400 MHz, CDCl₃) δ 8.62 (d, J = 15.2 Hz, 1H), 7.58 – 7.51 (m, 2H), 7.49 (t, J = 6.9 Hz, 2H), 7.41 (d, J = 9.2 Hz, 2H), 7.12 (dd, J = 6.2, 2.2 Hz, 2H), 6.99 (s, 1H), 6.84 (d, J = 15.3 Hz, 1H), 4.62 (q, J = 6.9 Hz, 2H), 2.77 (dt, J = 37.7, 5.5 Hz, 4H), 2.14 – 1.88 (m, 2H), 1.82 (s, 6H), 1.56 (t, J = 6.9 Hz, 3H).TOF HRMS: m/z calcd for C₂₈H₂₇F₃NO₄S⁺ [M⁺]: 530.1607, found: 530.1607.

Synthesis of Cy-B

106 mg 3 (0.20 mmol), 154 mg (0.6 mmol) Bis(pinacolato)diboron, 41 mg (0.50 mmol) NaOAc and 9 mg (0.01 mmol) Pd(dppf)Cl₂ was added in a flask. Then anhydrous toluene was bubbled with nitrogen for 30 min to remove the dissolved oxygen and introduced into the flask by injection under nitrogen atmosphere. The mixture was heated to 110 and stirred for 4 h. After cooling down to room-temperature, toluene was removed by distillation under reduced pressure and further purified by silica gel column chromatography with CH₂CH₂/CH₃OH (50:1) and get the royal purple solid (22 mg, 21.6%).¹H NMR (400 MHz, CDCl₃) δ 8.68 (d, J = 15.1 Hz, 1H), 7.67 (d, J =
7.6 Hz, 1H), 7.61 (d, J = 7.2, 2.7 Hz, 2H), 7.45 (d, J = 5.8 Hz, 2H), 7.35 (d, J = 7.6 Hz, 1H), 7.11 (s, 1H), 6.66 (d, J = 15.1 Hz, 1H), 4.52 (q, J = 7.2 Hz, 2H), 2.86 – 2.67 (m, 4H), 1.97 – 1.92 (m, 3H), 1.82 (s, 6H), 1.53 (t, J = 7.2 Hz, 3H), 1.39 (s, 12H);13C NMR (101 MHz, CDCl3) δ 177.83, 160.36, 152.29, 146.58, 142.25, 140.95, 131.88, 131.65, 131.49, 129.45, 127.86, 126.78, 124.09, 122.46, 121.14, 115.76, 113.11, 105.72, 84.59, 50.98, 41.33, 29.72, 28.12, 24.90, 24.86, 24.22, 20.11, 13.04 ppm; TOF HRMS: m/z calcd for C_{33}H_{39}BNO_{3}^[M]+: 508.3018, found: 508.3022.

**Figure S1** HRMS analysis of Cy-B before (a) and after (b) addition of H_{2}O_{2}

**Figure S2** a) Excitation spectrum of Cy-B in response to 100 μM H_{2}O_{2}. Emission wavelength: 709 nm. b) the ratio between the fluorescent intensity at 709 nm after excitation at 685nm and 554nm (I_{685}/I_{554}) before and after addition of 100 μM H_{2}O_{2}
**Figure S3** Photostability test of Cy-B before and after addition of H$_2$O$_2$ and traditional cyanine dye Cy-7 (compound 1)

**Figure S4** (a) UV-vis absorption changes of Cy-B (10μM) against different H$_2$O$_2$ concentrations (0, 2, 4, 6, 8, 10, 12, 14, 16, and 18 μM); (b) Fluorescence intensity changes at 709nm of Cy-B (10 μM) against different H$_2$O$_2$ concentrations (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140 and 150 μM)

**Figure S5** (a) UV-vis absorption changes and (b) Fluorescence intensity changes at 709nm of Cy-B (10μM) against 100 μM H$_2$O$_2$ at various pH in acetonitrile/phosphate buffer (1:9 v/v, 10 mM) system
Figure S6 Confocal images of exogenous H$_2$O$_2$ in HL-7702 cells. The excitation wavelength was 635 nm and the emission was collected at 700-750 nm. (a-c) Cells were stained with Cy-B (2.5 μM) for 15 min and then 30 μL PBS (pH 7.4, 0.01M) was added and incubated for 60 min. (d-f) Cells were stained with Cy-B (2.5 μM) for 15 min and then incubated with 100 μM H$_2$O$_2$ for 60 min. Scale bar = 20 μm

Figure S7 Confocal images of endogenous H$_2$O$_2$ in MCF-7 cells. The excitation wavelength was 635 nm and the emission was collected at 700-750 nm. (a-c) Cells were stained with Cy-B (2.5 μM) for 15 min and then incubated with 10 μL DMSO for 90 min. (d-f) Cells were stained with Cy-B (2.5 μM) for 15 min and then incubated with 5 μg/ml PMA for 60 min. Scale bar = 20 μm
Figure S8 Viability of MCF-7 cells in the presence of probe Cy-B as measured by using MTT assay. The cells were incubated with probe for 48 h
Figure S9 HRMS spectrum of 2

Figure S10 $^1$H NMR spectrum of 2 in CDCl₃.

Figure S11 HRMS spectrum of Cy-B.
Figure S12 $^1$H NMR spectrum of Cy-B in CDCl$_3$.

Figure S13 $^{13}$C NMR spectrum of Cy-B in CDCl$_3$.

References