Supplementary Information for

A Near-Infrared Fluorescent Aza-Bodipy Probe for Dual-wavelength Detection of Hydrogen Peroxide in Living Cells

Jingjing Xu, Jingying Zhai, Yanmei Xu, Jingwei Zhu, Yu Qin* and Dechen Jiang*

State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, China.

Phone: +86-25-83594846 (D.J) and +86-25-83592562 (Y.Q)

Email: dechenjiang@nju.edu.cn (D.J) and qinyu01@gmail.com (Y.Q)
1. Experimental section.

Reagents and Instrumentation.

Fluorescent probe azaBDPBA was synthesized following our previous report. HEPES free acid, sodium phosphate dibasic anhydrate, sodium phosphate monobasic monohydrate, ethanol anhydrous, sodium hydroxide and hydrogen peroxide (30%) solution were obtained from Sangon (Shanghai, China). Copper powder and 65% nitric acid were purchase from Wanqing (Nanjing, China). Tert-butyl hydroperoxide (70% solution in H$_2$O) (TBHP) and sodium hypochlorite (NaClO) (5% available chlorine) were purchase from J&K. Potassium superoxide was purchase from Alfa Aesar. Phorbol12-myristate 13-acetate (PMA) and catalase from bovine liver were purchased from Sigma. High-glucose Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Life technologies.

Perkin-Elmer LS50B fluorescence spectrometer was used for fluorescence measurement of azaBDPBA. Absorption spectra were recorded using α-1900 UV/Vis spectrometer (Puyuan instrument company, Shanghai). Leica TCS SP5 two-photon laser scanning confocal microscopy and Prism and Reflector Imaging Spectroscopy System (PARISS) with Olympus BX53 microscope were used to obtain the fluorescence imaging and spectra of azaBDPBA in HeLa cells. The pH was monitored with a calibrated glass pH electrode (Sartorius PB-10).

Homogeneous Solution Detection

All the fluorescent experiments in homogeneous phase were excited at 652 nm. The parameters were as follows: excitation slit width, 15 nm; emission slit width, 15 nm; scanning speed, 100 nm/min, 1% T attenuator. The fluorescence intensities at 724 and 682 nm were ratioed (F$_{724}$ nm/ F$_{682}$ nm) as the signal, and the standard deviation of F$_{724}$ nm/ F$_{682}$ nm from three independent measurements in 20 mM HEPES (pH 7.4) without hydrogen peroxide was taken as the noise. The concentration of hydrogen peroxide with the signal-to-noise ratio of 3 was determined as the detection limit.

For the selectivity to different reactive oxygen species (ROS), 20 mM HEPES (pH 7.4) containing 10 µM azaBDPBA and 5 mM different ROSs (unless otherwise stated) were prepared. Hydrogen peroxide, tert-butyldydroperoxide (TBHP), and sodium hypochlorite (NaOCl) were delivered from 30%, 70%, and 5% aqueous solutions respectively. Superoxide (O$_2^-$) was generated from solid K$_2$O$_2$. 100 mM hydroxyl radical (·OH) and tert-butoxy radical (·OBu) were generated
by reaction of 1 M Fe$^{2+}$ with 100 mM H$_2$O$_2$ or TBHP, respectively. NO was generated from the reaction between copper powder and 2 M nitric acid to obtain the final concentration of 60 mM. 10 mM hydrogen peroxide was scavenged by 10 units/mL catalase 30 min at 37°C. Peroxynitrite (ONOO$^{-}$) was synthesized according to the reported method$^2$. The concentration of peroxynitrite was estimated by using extinction coefficient of 1670 cm$^{-1}$ M$^{-1}$ at 302 nm.

**Preparation and Staining of Cell Cultures**

HeLa cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen). One day before the imaging, cells were passed and plated in 35 mm diameter confocal petri dish or plated with 0.17 mm thickness microscope cover glass. Before the experiments, cells were washed three times with PBS buffer (pH 7.4), incubated with 20 µM probe in medium, washed by PBS for three times and imaged.

**Fluorescence Imaging**

Prism And Reflector Imaging Spectroscopy System (PARISS) and Olympus BX53 microscope were used to obtain the fluorescence imaging and spectra (excited with green light) at HeLa cells with 40x objective lens.

Before the experiments, cells were incubated in the medium with aza-bodipy-BA (20 µM) for 30 min. After the washing of the cells with PBS, the imaging was performed. Then, the cells were exposed to the medium with different concentrations of hydrogen peroxide. Following the washing, the image process was re-taken.

Confocal fluorescence imaging was performed with a Leica TCS SP5 two-photon laser scanning confocal microscopy and 63x oil objective lens. Excitation of azaBDPBA loaded HeLa cells were carried out at 633 nm HeNe laser and emission was collected in two windows from 661 nm to 681 nm and 750 nm to 780 nm, respectively.

Aza-BODIPY-BA (20 µM) was incubated with live cell sample for 5 min 37 °C before addition of 10$^{-2}$ M H$_2$O$_2$. 
2. Titration curve of azaBDPBA to hydrogen peroxide

Figure S1. The titration curve relation $(F_{724\text{ nm}}/F_{682\text{ nm}})$ of azaBDPBA (10 µM) as a function of $H_2O_2$ concentrations.

3. Response of Location of azaBDPBA in the cells

Figure S2. Confocal fluorescence images of live HeLa cells incubated with 20 µM azaBDPBA for 5 min and Dio (green membrane fluorescence probe) for 2 h. (A) bright-field image; (B) fluorescence image for the location of Dio in cells; (C) fluorescence image for the location of azaBDPBA. (D) the overlay of image A, B and C.
Reference.
