Supporting Information for

Red emitting two-photon fluorescent probe for dynamic imaging of redox balance meditated by superoxide anion and GSH in living cells and tissues

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Synthesis and the response mechanism of the probe

![Fig. S1. Structures and synthetic route of probe NpRbH and the response mechanism of probe NpRbH.](image)

Synthesis of probe NpRbH

Compound 1 and donor 2 was synthesized according to our previous work\(^1,2\). Compound 1 (305 mg, 0.5 mmol), 3,4-dihydroxy-benzaldehyde (70 mg, 0.5 mmol), glacial acetic acid (0.2mL) and piperidine (1 mL) was dissolved in dry toluene (100 mL) in a Dean-Stark apparatus and then refluxed for 12 h. The solvent was concentrated under reduced pressure. The residue was purified by the silica gel chromatography (dichloromethane /ethyl acetate, \(20:1, \text{v/v}\)), which gave compound **NpRbH** as a dark-purple solid (36mg, 8%). \(^1\)HNMR (DMSO-d\(_6\), 400 MHz) \(\delta\) (ppm): \(\delta\) 9.49 (s, 1H), 9.36 (s, 1H), 8.63 (s, 1H), 8.23 (s, 1H), 8.12 (d, \(J = 8.11, 1H\)), 8.03 (m, \(J = 8.01, 3H\)), 7.88(m, \(J = 7.86, 3H\)), 7.55(d, \(J = 7.54, 2H\)), 7.46(d, \(J = 7.44, 2H\)),7.35 (m, \(J = 7.31, 2H\)), 7.11 (s, 1H), 7.03 (s, 1H), 6.96 (s, 1H), 6.91 (d, \(J = 6.90, 1H\)), 6.81 (d, \(J = 6.80, 1H\)), 6.20 (s, 1H), 3.10 (s, 6H), 1.99 (s, 3H), 1.52 (s, 3H), 1.47 (s, 3H).

MS (ESI): m/z 729.3 [M-H]\(^-\), calcld for C\(_{45}\)H\(_{37}\)BF\(_2\)N\(_4\)O\(_3\) 730.6.

Cell cytotoxic assays and two-photon fluorescence imaging

To evaluate the potential cytotoxicity of probe **NpRbH**, HeLa cells were seeded at \(1\times10^5\) cells per well in 96-well plates and incubated for 24 h and 48 h. After that different concentration (2-20 \(\mu\)M) of probe **NpRbH** was added to the cells and these cells were cultured for an additional 24 h or 48 h. And then the cytotoxic effects of **NpRbH** was
determined using MTT assays. Following incubation, the RAW264.7 cells were washed three times with Dulbecco's phosphate buffered saline (DPBS) and imaged. The two-photon excitation wavelength of the femtosecond laser was fixed at 780 nm; the emission wavelengths were recorded at 470-530 and 550-650 nm respectively.

**Two-photon fluorescence imaging of fresh mouse liver slices**

Fresh tissue slices were prepared from the livers of nude mice. The slices were incubated with analytes at 37 °C. The slices were washed with DPBS three times, and then two-photon fluorescence microscopy images were collected. The excitation wavelength of the femtosecond laser was set at 780 nm, the emission wavelengths were recorded at 470-530 and 550-650 nm, respectively.

![Fluorescence Emission Spectra](image)

**Fig. S2.** Fluorescence emission spectra. The fluorescence performance of NpRbH in the different organic phase ratio. NpRbH (5 μM) in PBS/EtOH (containing EtOH 10, 15, 20, 30%, pH= 7.4, 10 mM). λ_{ex} = 400 nm.

![Fluorescence Emission Spectra](image)

**Fig. S3.** Fluorescence emission spectra. The black and red lines represent donor 2 (5 μM), and NpRbH (5 μM) respectively, in PBS/EtOH (3/1, v/v, pH= 7.4, 10 mM), as the respective fluorescence responses; λ_{ex} = 400 nm.
Energy Transfer Efficiency (ETE) = \frac{[(fluorescence of donor-fluorescence of donor in cassette)/fluorescence of donor]}{fluorescence of donor} \times 100\%. \textsuperscript{1,2}

For NpRbH, ETE = \frac{(692056 - 59734)}{692056} \times 100\% = 91.37\%.

Fig. S4. (a) Time course of fluorescence change in detecting superoxide with NpRbH. (b) Photostability experiment of NpRbH before (red line) and after (black line) added O_2^-\textsuperscript{-}. In PBS/EtOH (3/1, v/v, pH= 7.4, 10 mM), as the respective fluorescence responses; \(\lambda_{ex} = 400\) nm, \(\lambda_{em} = 596\) nm.

Fluorescence titration and detection limit: The detection limit was determined from the fluorescence titration data. The fluorescence intensity increased linearly with the concentration of O_2^-\textsuperscript{-} ranging from 0.33 \(\mu\)M to 4.0 \(\mu\)M. The detection limit was calculated to be \(9.51 \times 10^{-8}\) M based on 3\(\sigma/slope\) method.

Fig. S5 The linear responses at low O_2^-\textsuperscript{-} concentrations (0-4 \(\mu\)M). \(\lambda_{ex} = 400\) nm.
The reversibility of NpRbH

The proposed reaction mechanism of NpRbH with O$_2^{-}$ was shown in Figure S1. The structure of NpRbO was confirmed by MS (ESI): m/z 729.5 [M+H]$^+$, calcld 728.3. The fluorescence responses of NpRbH to different concentrations of GSH were showed in Figure S5. The fluorescence of NpRbH increases gradually with the increase of GSH concentrations, until the [GSH] reaches 2 mM. So 1.5 mM GSH was used to convert NpRbH into NpRbO in the reversible cycle experiment. The reversibility of the probe was tested (Figure S6). This reversible cycle can be repeated for three times more under the same conditions. The reversibility implied the advantage of NpRbH for dynamic determine O$_2^{-}$ in cells and in vivo.

**Fig. S6.** The fluorescence emission spectra of NpRbO (5 μM) in the presence of different concentrations of GSH (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2 mM) in PBS buffered (10mM, pH=7.4) aqueous EtOH solution (3:1, v/v). $\lambda_{ex} = 400$ nm.

**Fig. S7.** The reversibility test of NpRbH for detection O$_2^{-}$ and GSH. NpRbH was added with 25 μM O$_2^{-}$, after 10 min, the solution was treated with 1 mM GSH. When the fluorescence returned to the baseline level, another 25 μM O$_2^{-}$ was added to the mixture after 10 min. $\lambda_{ex} = 400$ nm, $\lambda_{em} = 596$ nm.
**Fig. S8.** Effect of pH on the fluorescence intensity of NpRbH (5 μM) in buffered/EtOH (3/1, v/v, pH= 4-9, 10 mM). Fluorescence responses are shown before (■), after (●) addition of O$_2$•− (25 μM) and further addition of GSH (1mM) (▲), respectively. Where F$_0$ represents the fluorescence intensity of NpRbH (5 μM), the curve was plotted with the fluorescence intensity at 596 nm vs pH value. $\lambda_{ex}$ = 400 nm.

**Fig. S9.** Fluorescence responses of 5 μM NpRbH to various metal ions. The fluorescence intensity at $\lambda_{em}$=596 nm was plotted versus substances: 1-10. Mn$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Mg$^{2+}$, Ca$^{2+}$ (500 μM), O$_2$•− (25μM). Data were acquired in buffered/EtOH (3/1, v/v, pH= 7.4, 10 mM), with $\lambda_{ex}$ = 400 nm.

**Fig. S10** TP absorption cross-section of NpRbH and NpRbO in PBS buffered (10 mM, pH = 7.4) aqueous EtOH solution (3:1, v/v). Black line represents the active absorption cross-section of NpRbH and red line represents the active absorption cross-section of NpRbO.
Fig. S11. Cytotoxicity of both NpRbH against HeLa cells as determined by MTT assay: HeLa cells were treated with NpRbH (0-20 μM). Black bar and red bar represents incubation for 48 h and 24 h respectively.

Fig. S12. TP fluorescence images of O$_2^\cdot$•/GSH reversible cycles in live RAW264.7 cells with NpRbH (2.5 μM). (a) Cells incubated with only probe NpRbH for 30 min.; (b) Cells were pretreated with PMA (5.0 μg/mL) for 30 min. Green channel $\lambda_{em}$=470-530 nm, red channel $\lambda_{em}$=550-650 nm. The results demonstrated that fluorescence signal coming from the donor remained very weak in the green channel due to the effective TBET process of the probe.$^2$
Fig. S13. Depth TP fluorescence images of: (a) NpRbH (5 μM) in tissues (0-300 μm); (b) After added PMA (5 μg/mL) in tissues (0-300 μm) and (c) After treatment with PMA, flowed
with GSH (1mM) for another 60 min in tissues (0-300 μm). Step size: 3 μm for a, c and 2 μm for b. Scale bars: 200 μm. $\lambda_{ex}$=780nm, red channel $\lambda_{em}$=550-650 nm.

References


Mass Spectra and $^1$HNMR
Fig. S14 $^1$HNMR spectrum of the compound NpRbH

Fig. S15 ESI mass spectrum of the compound NpRbH.
Fig. S16 ESI mass spectrum of the compound NpRbO.