Supporting Information

A novel ratiometric fluorescent probe for highly sensitive and selective detection of peroxynitrite and its application for tracing endogenous peroxynitrite in live cells

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1. Materials and instruments

Except for special labels, chemical reagents were obtained from commercial vendor and employed without further purification. High resolution mass spectra (HRMS) were obtained by LC-MS2010A instrument. ^{1}H and ^{13}C NMR data were obtained by Bruker AV-400 NMR spectrometer. Absorption spectra were obtained by UV-3101PC spectrophotometer. Fluorescence spectra were obtained by Horiba FluoroMax-4 spectrophotometer. Fluorescence imaging of $ONOO^-$ in live RAW 264.7 macrophage cells and zebrafish were carried out on an Olympus FV1000-IX81 confocal fluorescence microscope.

2. Determination of the detection limit

The detection limit was calculated based on the fluorescence titration. The fluorescence spectra of free probe **CPD-ratio** were measured by five times and its standard deviation was obtained. To gain the slope, the fluorescence intensity ratios (at 500 nm / 565 nm) were plotted as the increasing concentrations of $ONOO^-$, so the detection limit was calculated with the following equation (1):

$$\text{Detection limit} = 3\sigma/k \qquad (1)$$

Where σ is the standard deviation of blank measurement, k is the slope between the fluorescence intensities ratios versus the concentrations of $ONOO^-$.

3. Cytotoxicity assays

The cell viability of RAW 264.7 macrophage cells, treated with probe **CPD-ratio**, was assessed by a cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Tokyo, Japan). Briefly, RAW 264.7 macrophage cells, seeded at a density of 1×10^6

cells·mL^{-1} on a 96-well plate, were maintained at 37 °C in a 5% CO_2 / 95% air incubator for 12 h. Then the live RAW 264.7 macrophage cells were incubated with various concentrations (0, 2, 5, 10, 20, and 30 μM) of probe **CPD-ratio** suspended in culture medium for 12 h. Subsequently, CCK-8 solution was added into each well for 2 h, and absorbance at 450 nm was measured.

4. Imaging studies of live cells

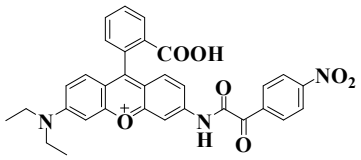
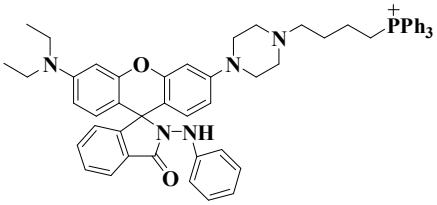
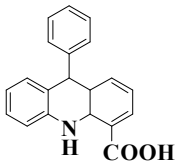
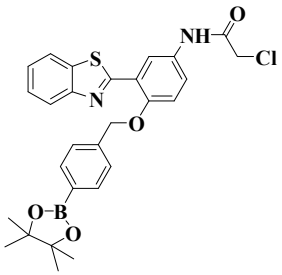
The RAW 264.7 macrophage cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin-streptomycin and incubated under an atmosphere containing 5% CO_2 at 37 °C humidified air for 24 h. Before imaging by confocal fluorescence microscope, probe **CPD-ratio** (10 μM) was used as a bioimaging reagent to incubate RAW 264.7 macrophage cells for 30 min, then removed culture medium and washed with phosphate-buffered saline for three times. And cells incubated with probe **CPD-ratio** (10 μM) for another 30 min after preincubation with 4-amino-tempo (200 μM). After that, these probe-loaded cells were further incubated upon addition of $ONOO^-$ (20 μM) for 30 min. On the other hand, the cells pretreated with PMA (1.0 μg mL^{-1}) or LPS (1.0 μg mL^{-1}) for 1 h, then were incubated with probe **CPD-ratio** (10 μM) in culture media for another 30 min, and washed with culture water. Then the fluorescence imaging of cells was carried out by confocal fluorescence microscope.

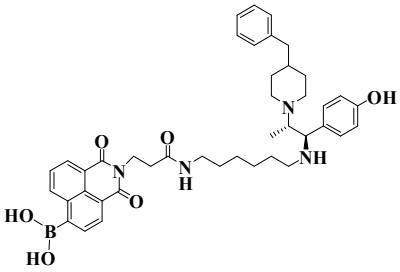
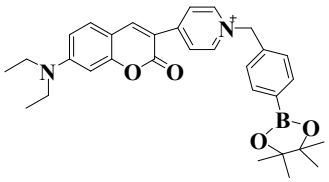
5. Preparation of reactive oxygen species (ROS) and reactive nitrogen species (RNS)

Hydrogen peroxide (H_2O_2), sodium hypochlorite (NaOCl), and *tert*-butylhydroperoxide (TBHP) were diluted from the commercially available solution to

0.1 M in ultrapure water. Hydroxyl radical ($^{\cdot}$OH) and *tert*-butoxy radical ($^{\cdot}$O^{t}Bu) were generated by Fenton reactions. Superoxide (O_2^{-}) was prepared from KO_2 in DMSO. Singlet oxygen ($^1O_2$) was generated from HOCl and H_2O_2. Nitric oxide (NO) was generated from potassium nitroprusside dihydrate. The concentration of H_2O_2 was determined from the absorption at 240 nm (ε = 43.6 M^{-1} cm^{-1}). The concentration of OCl^- was determined from the absorbance at 292 nm (ε = 350 M^{-1} cm^{-1}). $ONOO^-$ was prepared according to the reported method and the concentration was determined based on the absorbance at 302 nm (1670 M^{-1} cm^{-1}).

6. Additional table of comparison between reported $ONOO^-$ probes and probe CPD-ratio

Probe	λ_{em}	Time	Detection limit	Imaging	References
	558 nm	30 min	43 nM	Living cells	Anal. Chem. 89 (2017) 7693-7700
	578 nm	20 min	53 nM	Living cells	Anal. Chem. 89 (2017) 5519-5525
	496 nm	5 s	16 nM	Living cells	ACS Sens. 2 (2017) 501-505
	405/481 nm	1 min	21.4 nM	Living cells	Chem. Commun. 54 (2018) 9953-9956

	450/550 nm	200 s	184 nM	Living cells and tissues	Anal. Chem. 90 (2018)9347-9352
	500/565 nm	1 min	47 nM	Living cells	This work

7. The HRMS data for CPD-ratio and its reaction products with $ONOO^-$

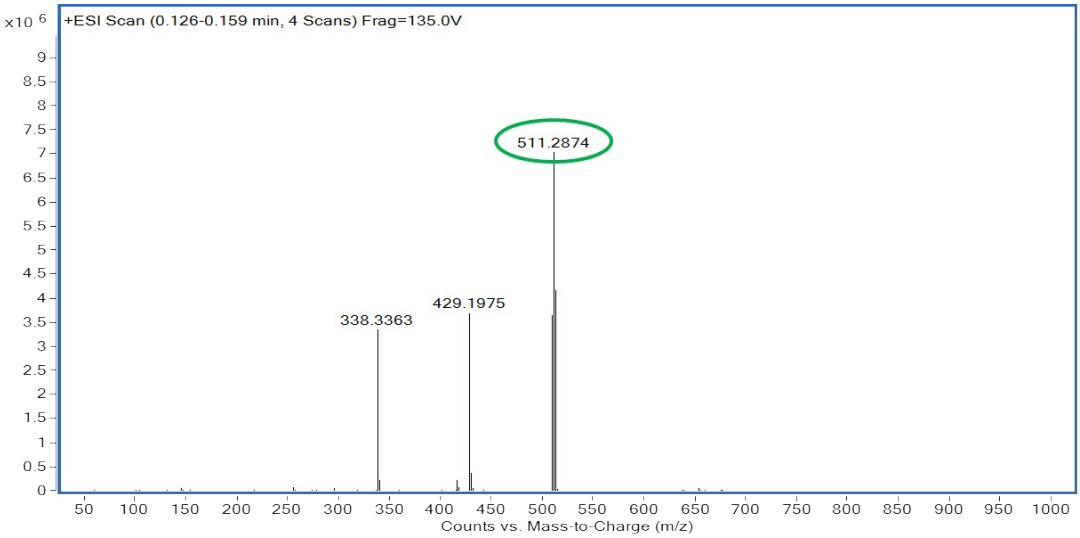


Figure S1. HRMS data of probe **CPD-ratio**.

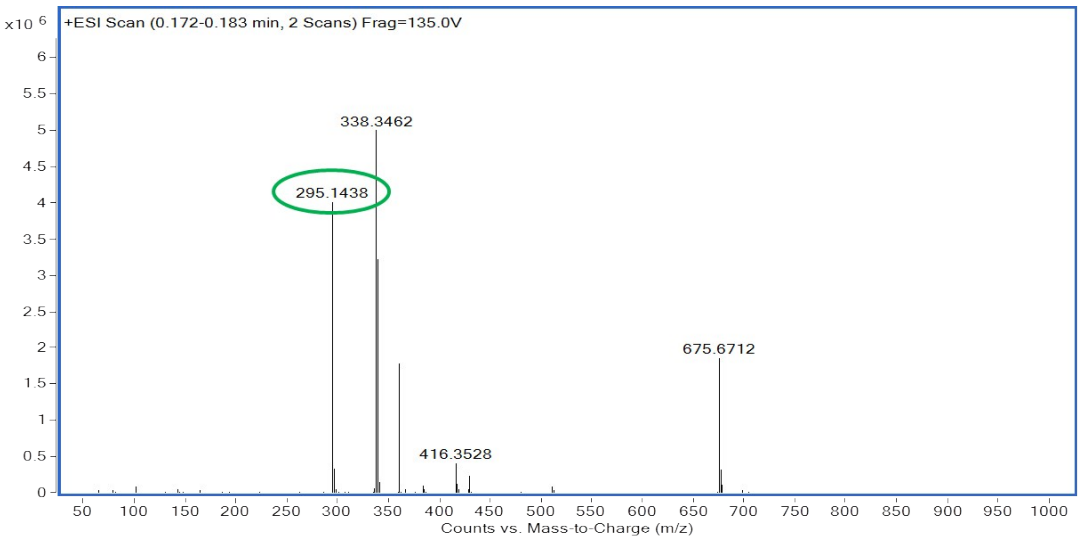


Figure S2. HRMS data of the reaction products of probe **CPD-ratio** and $ONOO^-$.