Supporting information for

# A Near-Infrared Reversible Fluorescent Probe for Peroxynitrite and Imaging of Redox Cycles in Living Cells

*Kehua Xu, Huachao Chen, Jiangwei Tian, Baiyu Ding, Yanxia Xie, Mingming Qiang and Bo Tang**

## Contents:

## 1. Apparatus and Materials

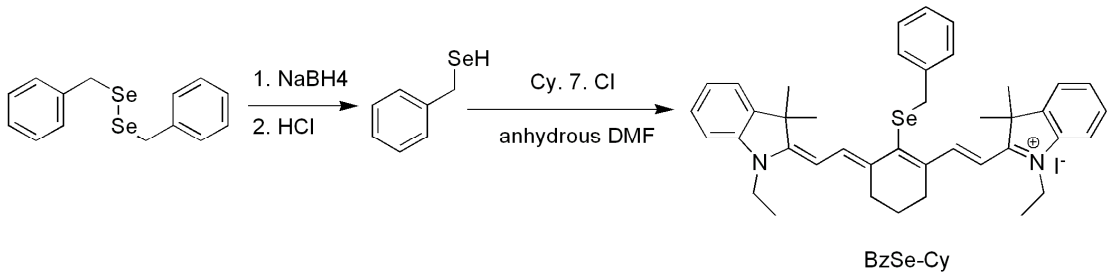
**Apparatus.** Fluorometric traces were obtained with a Cary Eclipse fluorescence spectrophotometer (Varian, Inc., USA) with a 1.0 cm quartz cells at the slits of 10/10 nm. Absorption spectra were measured on a pharmaspect UV-1700 UV-visible spectrophotometer (SHIMADZU). Ozone generator was purchased from Beyok Electronic Co., Zhejiang, China. All pH measurements were performed with a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode. $^{1}H$, $^{13}C$, and $^{77}Se$ NMR spectra were taken on Bruker Advance 600-MHz and Bruker 300-MHz spectrometer. The fluorescence images of cells were taken using a LTE confocal laser scanning microscope (Germany Leica Co., Ltd) with an objective lens (×40). Absorbance was measured in a TRITURUS microplate reader in the MTT assay. Determination of organic elements was obtained with Model PE-2400(II) element analyzer.

**Materials.** Unless stated otherwise, solvents were dried by distillation. All reagents were of commercial quality and used without further purification. 2-[4-Chloro-7-(1-ethyl-3,3-dimethyl(indolin-2-ylidene)]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl)-1-ethyl-3,3-dimethyl-3H-indolium (Cy.7.Cl) was synthesized in our laboratory.[1] Cysteine (Cys), glutathione (GSH), propidium iodide (PI), 4',6-diamidino-2-phenylindole (DAPI), Mito Tracker Green FM, SIN-1 (3-morpholinosydnonimine, a model compound for the continuous release of NO, $O_2^{\cdot-}$), S-nitroso-N-acetyl-dl- penicillamine (SNAP), xanthine (1 mm) in 10 mm NaOH

solution, xanthine oxidase (5 $UmL^{-1}$), $H_2O_2$, 3-(4, 5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company. Dibenzyl diselenide (95+%) and SNP (Sodium Nitroferricyanide (III) Dihydrate) were purchased from Alfa Aesar Chemical Company. Ascorbic acid and dithiothreitol (DTT) were obtained from Sinopharm. Chemical Regent Co., Ltd. Sartorius ultrapure water (18.2 MΩ cm) water was used throughout the analytical experiments. RAW 264.7 cells were purchased from the Committee on Type Culture Collection of the Chinese Academy of Sciences.

**2. Synthesis of BzSe-Cy**

According to the synthesis of a series of near-infrared cyanine dyes proposed by Xiaojun Peng,[2] with some modification, we synthesized the new probe BzSe-Cy ( Scheme S1). Solid $NaBH_4$, (0.26 g, 6.9 mmol) was added over a 15-min period to a suspension of dibenzyl diselenide (1.021 g, 3 mmol) in 60 mL of absolute ethanol cooled to 0 ºC under a stream of nitrogen. After completion of the addition, the mixture was allowed to warm to room temperature and stirred 30 min to give a clear solution. This reaction was quenched with 20 mL of 5% HCl and extracted with 3×10 mL of 1: l ether/pentane. The organic phase was dried over anhydrous sodium sulfate and the solvent was evaporated. The benzylselenol was given as colorless foul-smelling oil. DMF (15 mL) was added to benzylselenol, and then cyanine (0.196 g) was added to the solution. The mixture was stirred at 43 ºC under Ar for 48 h. Finally, the mixture was purified on silica gel chromatography eluted with ethyl acetate/methanol (4:1 v/v) to give BzSe-Cy as a green solid (56% yield).




BzSe-Cy

**Scheme S1.** Synthesis of BzSe-Cy

## 3. Characterization of BzSe-Cy

$^{1}$H NMR, $^{13}$C NMR, $^{77}$Se NMR spectra were recorded on a Bruker Advance 600 MHz and Bruker 300 MHz spectrometer. Chemical shifts were collected in Acetone-$d_6$. $^{1}$HNMR (600 MHz, Acetone-$d_6$, 25 °C, TMS) δ(ppm): 8.80 (d, *J*=13.8 Hz, 2H), 7.60 (d, *J*=7.2 Hz, 2H), 7.40-7.47 (m, 4H), 7.29 (t, *J*=7.2 Hz, 2H), 7.25 (d, *J*=7.8 Hz, 2H), 7.16-7.23 (m,3H), 6.37 (d, *J*=14.4 Hz, 2H), 4.31 (q, *J*=6.6 Hz, 4H), 4.12 (s, 2H), 2.71 (t, *J*=6.6 Hz, 4H), 1.86 (m, 2H), 1.71 (s, 12H), 1.42 (t, *J*=6.0 Hz, 6H). $^{13}$C-NMR δ(ppm): 206.07, 172.95, 158.50, 150.14, 143.03, 142.15, 139.95, 134.98, 129.57, 129.56, 129.31, 127.92, 125.87, 123.34, 111.62, 101.75, 50.06, 39.92, 34.81, 27.91, 27.18, 21.87, 12.54. $^{77}$Se-NMR δ(ppm): 265; MS: m/z Calcd 647.9, found 647.5 $[M]^{+}$. Elemental Analysis: Calcd C, 63.6; H, 6.1; N, 3.6. Found C, 63.7; H, 6.2; N, 3.7.

## 4. Absorption and Emission Spectra of BzSe-Cy

Fluorescence emission spectra were obtained with a 1.0-cm quartz cells. A solution of 20 μM BzSe-Cy in 30 mM PBS (final concentration) at pH 7.4 containing 5% $CH_3CN$ as a cosolvent was prepared, and the bioanalytes were added to the mixture before measurement. The fluorescence intensity was measured in real-time at $\lambda_{ex}/\lambda_{em}$ =

770/800 nm. All performs were made in the presence of 0.10 M NaCl to maintain a constant ionic strength.

Absorption and emission spectra of 20 μM BzSe-Cy were recorded at 37 °C in PBS (pH 7.4, final concentration: 30 mM) (Fig. S1). The probe shows that $\lambda_{max}$ of excitation and emission lies in 770 and 800 nm, respectively. Stokes shift is 30 nm.

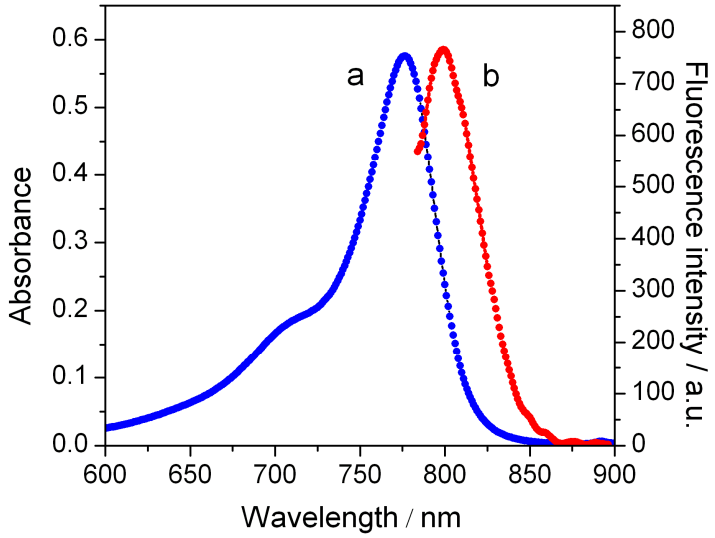



**Fig. S1**. a) Absorption and b) emission spectra of BzSe-Cy, $\lambda_{ex}/\lambda_{em}$ = 770/800 nm.

## 5. The pH Dependence of the Probe Reaction

Because pH of the buffer solution plays an important role in the sensitivity of $ONOO^-$ determination, we monitored the effect of pH on fluorescence intensity in the range of 6.0~8.0, as shown in Fig. S2. The fluorescence of the probe is relatively steady as the pH rised from 6.0 to 8.0. However, when we added 5 μM $ONOO^-$ to the solution, the fluorescence intensity gradually decreased as the pH rised from 6.6 to 7.2 and reached a constant value since then. This is because $ONOO^-$ is pH dependent (ONOOH, pKa=6.8).[3] In order to apply in biological system, pH=7.4 was selected

for the following determination system.

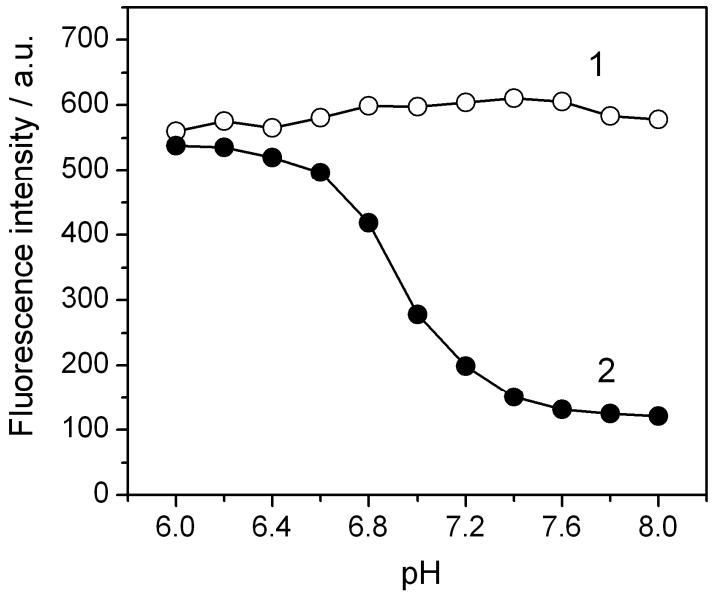



**Fig. S2**. Effects of pH on the reaction between BzSe-Cy (5 μM) and $ONOO^-$. Line 1: 0 μM $ONOO^-$, phosphate buffer; Line 2: 5 μM $ONOO^-$ , phosphate buffer.

## 6. Fluorescence Quantum Yield ($\Phi_F$) and Oxidation Potential of BzSe-Cy

**Fluorescence Quantum Yield ($\Phi_F$).** For measurement of the quantum yield of BzSe-Cy, the solution of the probe was adjusted to an absorbance of ~ 0.05. The emission spectra were recorded using 720 nm as excitation wavelength, and the integrated areas of the fluorescence-corrected spectra were measured. Relative fluorescence quantum yield ($\Phi_F$ = 0.106) were obtained by comparing the area under the emission spectrum of the test samples with that of a solution of IR-786 in methanol ($\Phi_F$ = 0.159).[4]

**Oxidation Potential.** Cyclic voltammetry was performed on an LK2005 electrochemical workstation (Tianjin Lanlike Chemistry & Electron High Technology

Co. Ltd., China). A three-electrode arrangement in a single cell was used for the measurements: a Pt wire as the auxiliary electrode, a Pt electrode and a GC electrode as the working electrode, and an Ag/AgCl (sat. NaCl) electrode as the reference electrode. Sample solutions contained 1 mM BzSe-Cy and 0.1 M sodium phosphate as a supporting electrolyte. The oxidation potential of BzSe-Cy is 0.622 V.

## 7. Test for BzSeO-Cy by ESI-MS and $^{77}$Se-NMR

We also assessed the structure of BzSeO-Cy. The probe BzSe-Cy was dissolved in $CH_3CN$ and then diluted to 20 μM with 0.1 M PBS buffer at 7.4. Then 5 μM $ONOO^-$ was injected into the probe solution. The mixture was stirred at 37 °C for 5 min. The solution was concentrated by evaporating the organic solvent. The reaction product was analyzed by ESI-MS. The ESI-MS spectrum of the reaction solution exhibited a peak at m/z 647 corresponding to the BzSe-Cy and a peak at m/z 663 corresponding to BzSeO-Cy (Fig. S3). Additionally, selenium is very sensitive to its electronic environment and possesses a large chemical shift range (approximately 3400 ppm).[5] These characteristics make selenium an excellent nucleus for NMR research. So we use $^{77}$Se NMR spectroscopy to further identify the structure of BzSeO-Cy. First, we analysed the pure BzSe-Cy solution using $^{77}$Se NMR. Fig. S4a shows the $^{77}$Se NMR spectrum of BzSe-Cy in $CD_3CN$ at 25 °C. There was only one single peak in the spectral range of 0-1200 ppm, and the chemical shift 265 ppm is in the range of the divalent selenium.[6] We next used $^{77}$Se NMR to analyze the reaction solution of the probe with $ONOO^-$. A new peak at 908 ppm was obtained, which is in the range of the quadrivalent selenium as shown in Fig. S4b.[7] So we can draw the conclusion that

the selenium atom in the probe has already been oxidized to quadrivalent selenium by $ONOO^-$.

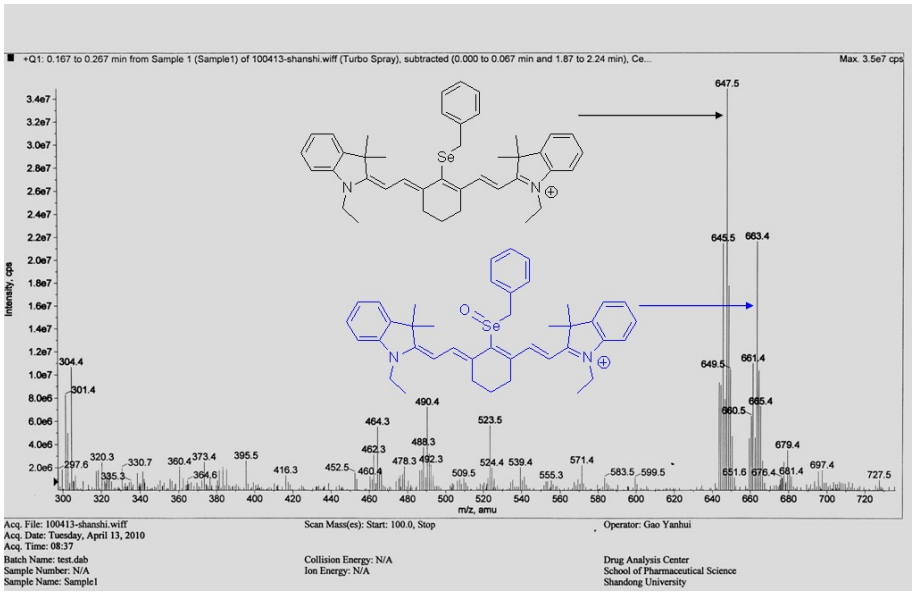



**Fig. S3.** Mass spectrum of the reaction solution of BzSe-Cy plus $ONOO^-$.

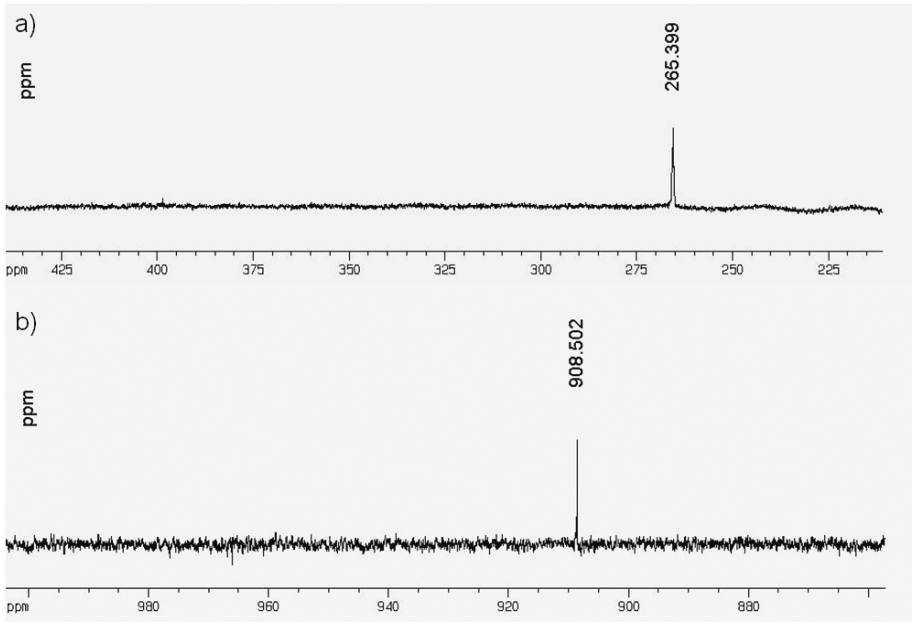



**Fig. S4.** $^{77}Se$ NMR spectra of a) BzSe-Cy and b) BzSeO-Cy at room temperature.

## 8. Preparation of ROS and RNS

The concentration of the stock $H_2O_2$ solution was titrated according to iodometry. $^1O_2$ was generated by the reaction of $H_2O_2$ with NaClO. $O_2^{\cdot-}$ was created by the enzymatic reaction of xanthine/xanthine oxidase (XA/XO) at 25 °C for 5 min. The experiments were performed under anaerobic conditions.[8] Deionized water was degassed with Ar for 20 min. NO was generated from SNP. SNP was added into degassed deionized water under Ar atmosphere then stirred for 30 min at room temperature. The probe solution was also degassed before the reaction with NO. ROO· was generated by AAPH. ·OH was generated by Fenton reaction ($Fe^{2+}$ + $H_2O_2 \rightarrow Fe^{3+}$ + ·OH + $OH^-$).[9] Commercial bleach was the source of NaClO, and the concentration of $ClO^-$ was determined by titration with $S_2O_3^{2-}$. The source of $NO_2^-$ was sodium nitrite and $NO_3^-$ was potassium nitrate. $ONOO^-$ was prepared as reported and was frozen at less than -20 °C.[10] The $ONOO^-$ solution prepared was usually very basic (pH 12). When larger volumes of $ONOO^-$ were added, part of the excess base was neutralized on the day of the experiment. The concentration of $ONOO^-$ solution was estimated by using an extinction coefficient of 1670 $cm^{-1}M^{-1}$ at 302 nm at the beginning and the end of the study to ascertain that $ONOO^-$ had not decomposed during the experiments. Ozone solutions were prepared in PBS buffer (pH = 6.0) and the concentrations were determined by UV absorption ($\lambda_{max}$ = 258 nm; ε = 2900l $mol^{-1}$ $cm^{-1}$).[11]

## 9. Cell Culture and Confocal Imaging

RAW 264.7 cells are a macrophage-like cell line derived from BALB/c mice

transformed by Abelson leukemia virus. RAW 264.7 cells were maintained following protocols provided by the American Type Tissue Culture Collection. Cells were seeded at a density of $1\times10^6$ cells $mL^{-1}$ in high glucose Dulbecco's Modified Eagle Medium (DMEM, 4.5 g of glucose/L) supplemented with 10% fetal bovine serum (FBS), $NaHCO_3$ (2 g/L) and 1% antibiotics (penicillin /streptomycin, 100 U/mL). The cells were maintained in a humidified incubator at 37 °C, in 5% $CO_2$/95% air. One day before imaging, cells were passed and plated on 18-mm glass coverslips in culture dish. Confocal fluorescence imaging studies were performed with a LSM510 confocal laser scanning microscope (Germany Leica Co., Ltd) with an objective lens (×40). A solution of 20 μM BzSe-Cy in culture solution containing 5% $CH_3CN$ as a cosolvent was prepared before conflcal imaging. Excitation of BzSe-Cy-loaded cells at 633 nm was carried out with a HeNe laser, and emission was collected using a META detector between 700 and 800 nm. Prior to imaging, the medium was removed. Cell imaging was carried out after washing cells with PBS (pH 7.4, 0.10 M) for three times.

**10. Test for the selectivity of BzSe-Cy in living cells**

The selectivity of this probe has been further assessed *in vivo*. We monitored the fluorescence images from treated with different oxidants, such as SIN-1, SNAP (NO donor), and XA/XO (superoxide donor). The RAW 264.7 cells were incubated with BzSe-Cy (20 μM) for 30 min and then washed three times with PBS buffer. As expected, the cells showed bright fluorescence in the absence of stimulants (Fig. S5a). A marked decrease of fluorescence in the living cells was induced after treatment with SIN-1 (20 μM) (Fig. S5b), but not with either SNAP (Fig. S5c) or XA/XO (Fig. S5d).

So we can draw a conclusion that BzSe-Cy can be used for the selective detection of $ONOO^-$ in living cells.

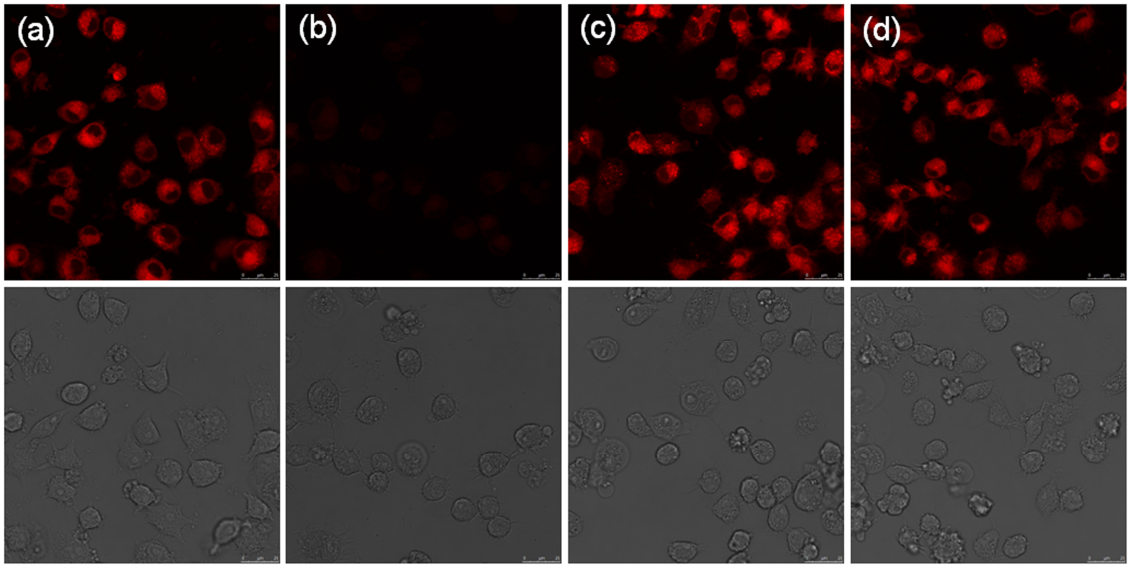



**Fig. S5.** Fluorescence imaging of RAW 264.7 cells. The cells were incubated with BzSe-Cy (20 μM) for 30 min and then subjected to different treatments. (a) Control. (b) 20 μM of SIN-1. (c) 20 μM of SNAP. (d) 100 μM of XA and 0.1 IU of XO.

## 11. Relationship between Stimulation Time and Fluorescent Brightness in RAW 264.7 Cells

To ascertain the relationship between the amount of $ONOO^-$ produced and stimulation time in living cells, the dependence of fluorescence intensity upon SIN-1-stimulating time was investigated (Fig. S6). First, a control experiment in cells without SIN-1 stimulation has been performed. As shown in Fig. S6a-c, the fluorescence of this probe remains relatively stable in living cells without SIN-1 during 60 min. Next, stimulation experiments were carried out at 20 min, 40 min, 60 min, respectively. Subsequently, the change trend of fluorescent brightness and cell

morphology were evaluated. Images revealed that fluorescence of Fig. S6h and Fig. S6i were obviously weaker than that of Fig. S6g. The imaging results showed that the amount of $ONOO^-$ produced is related with stimulation time in living cells, and it was appropriate to stimulate for 60 min with SIN-1.

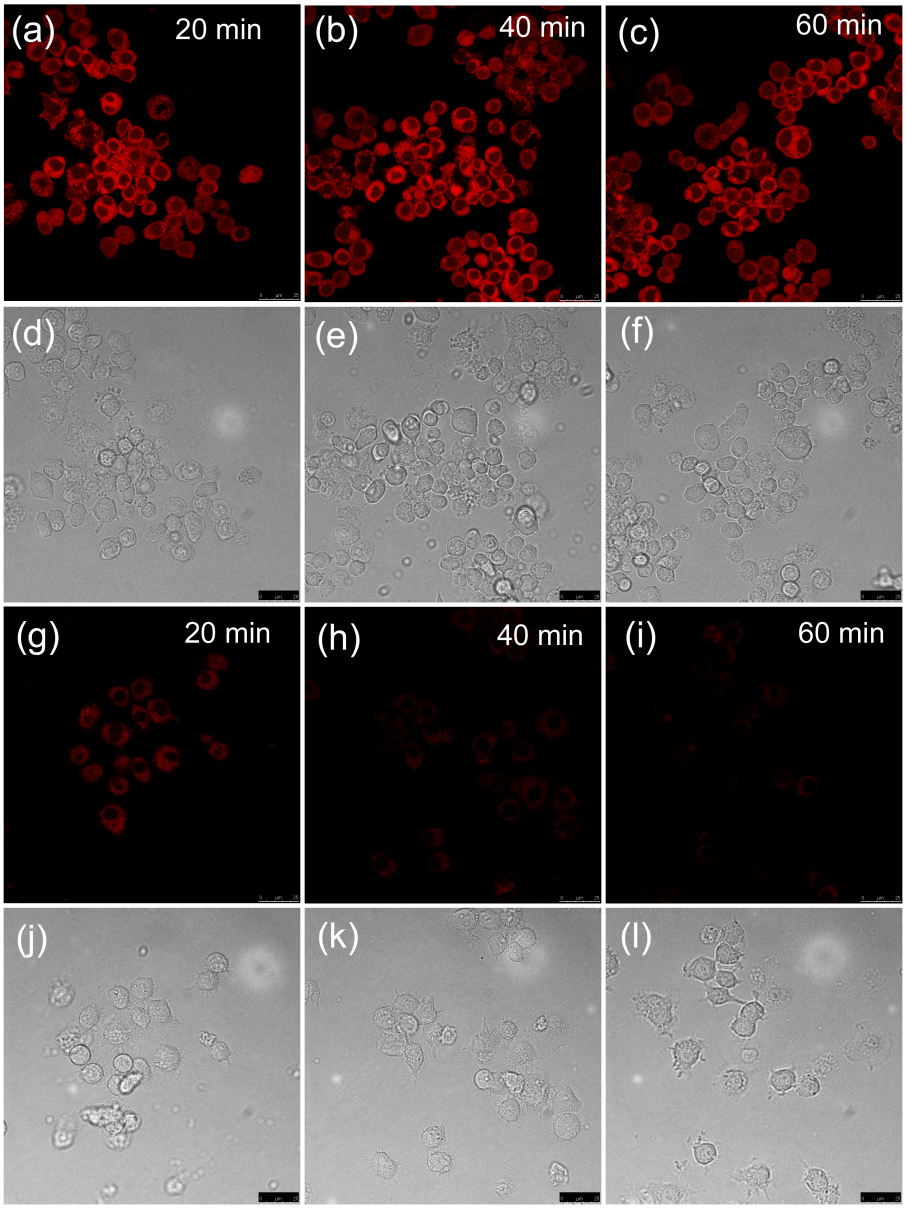



**Fig. S6.** Fluorescence imaging of probe-loaded RAW 264.7 cells after stimulation with 0 μM SIN-1 for 20 min, 40 min and 60 min. a)-c) fluorescence images, d)-f) bright-field images; Fluorescence imaging of probe-loaded RAW 264.7 cells after

stimulation with 20 μM SIN-1 for 20 min, 40 min and 60 min. g)-i) fluorescence images, j)-l) bright-field images.

**12. Test for Cell Membrane Permeability of BzSe-Cy**

To study the cell membrane permeability of BzSe-Cy, the interactions of the cells with probe at 4 °C and 37 °C have been investigated, respectively. Fig. S7a showed that when RAW 264.7 cells were incubated with 20 μM BzSe-Cy for 30 min at 37 °C, the bright fluorescence appeared. Fig. S7b is bright-field image of a, and Fig. S7c is merged images of a and b. This phenomenon suggests that BzSe-Cy can be easily internalized by the living cells. However, it is insufficient to prove that BzSe-Cy exhibits cell membrane permeability. As we know, the cell membrane permeability means that molecules can enter into cells by passive diffusion (transduction) rather than active transport (endocytosis).[12] Transduction but endocytosis is an energy-independent process unaffected at low temperatures.[13] Thus, to verify transduction, we examined the intracellular delivery of BzSe-Cy at 4 °C. RAW 264.7 cells were incubated with the probe at 4 °C to minimize energy dependent endocytic pathways and mitigate uptake of BzSe-Cy effectively at this temperature. As shown in Fig. S7d, the intracellular fluorescence was still apparent, confirming that the internalization of this probe was not substantially reduced at low temperature and transduction was involved in the intracellular delivery of BzSe-Cy. Bright-field transmission measurements after BzSe-Cy incubation confirmed that the cells were viable (Fig. S7e). Merged images of confocal laser scanning and optical microscopy images were shown in Fig. S7f. All of these experiment results indicated that the

probe exhibits excellent cell membrane permeability and is suitable for fluorescent imaging in biological samples.

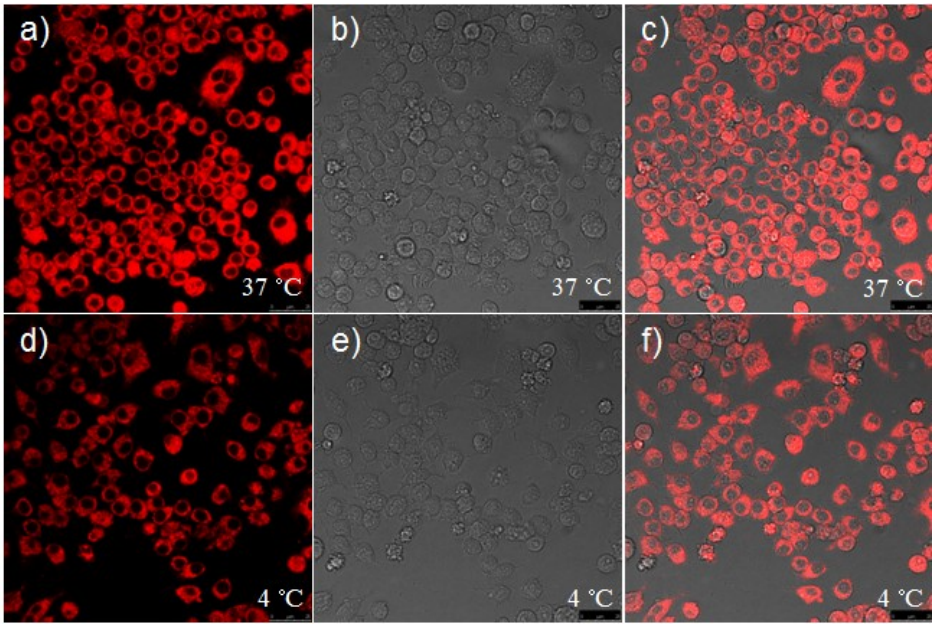



**Fig. S7**. Confocal fluorescence images of living RAW 264.7 cells. BzSe-Cy internalization was studied at 4 °C to mitigate energy dependent endocytic pathways. a) Cells were incubated with 20 μM BzSe-Cy for 30 min at 37 °C. b) bright-field image of a). c) merged images of red a) and b) bright-field channels. d) Cells were incubated with 20 μM BzSe-Cy for 30 min at 4 °C. e) bright-field image of d). f) merged images of red d) and e) bright-field channels.

## 13. Test for Cytotoxicity of BzSe-Cy

**MTT Assay.** To investigate BzSe-Cy cytotoxicity, MTT assay were carried out when the probe existed macrophages. RAW 264.7 cells ($10^6$ cell $mL^{-1}$) were dispersed within replicate 96-well microtiter plates to a total volume of 200 μL $well^{-1}$. Plates were maintained at 37 °C in a 5% $CO_2$/95% air incubator for 4 h. The probe was

diluted to different concentrations of solution with medium and added to each well after the the original medium has been removed. Macrophages were incubated with probe concentrations for 4 h. The concentrations of the probe were 1 μM to 1000 μM. And then 200 μL MTT solution (5.0 mg $mL^{-1}$, PBS) was added to each well. After 4 h, the remaining MTT solution was removed, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 490 nm in a TRITURUS microplate reader (Fig. S8). Calculation of $IC_{50}$ values was done according to Huber and Koella.[14] The probe $IC_{50}$ value was calculated to be 912 μM, which demonstrated that BzSe-Cy should be a low cytotoxic probe under experimental conditions at the concentration of 20 μM.

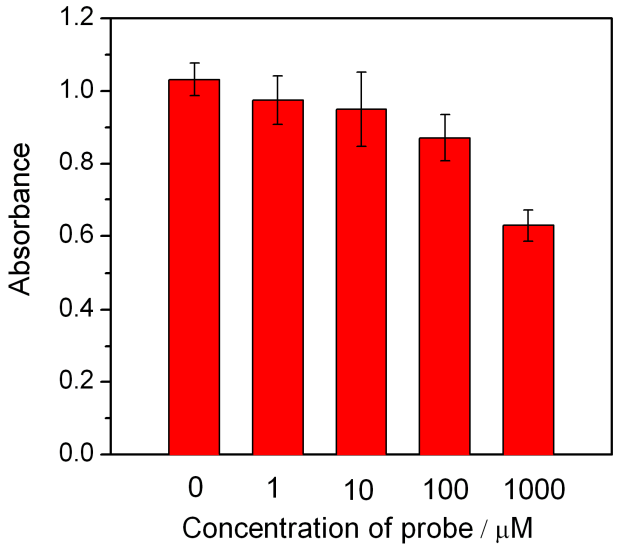



**Fig. S8**. MTT assay of macrophages in the presence of different concentrations of BzSe-Cy.

## 14. $^{1}$H-NMR, $^{13}$C-NMR, MS and IR spectra of BzSe-Cy

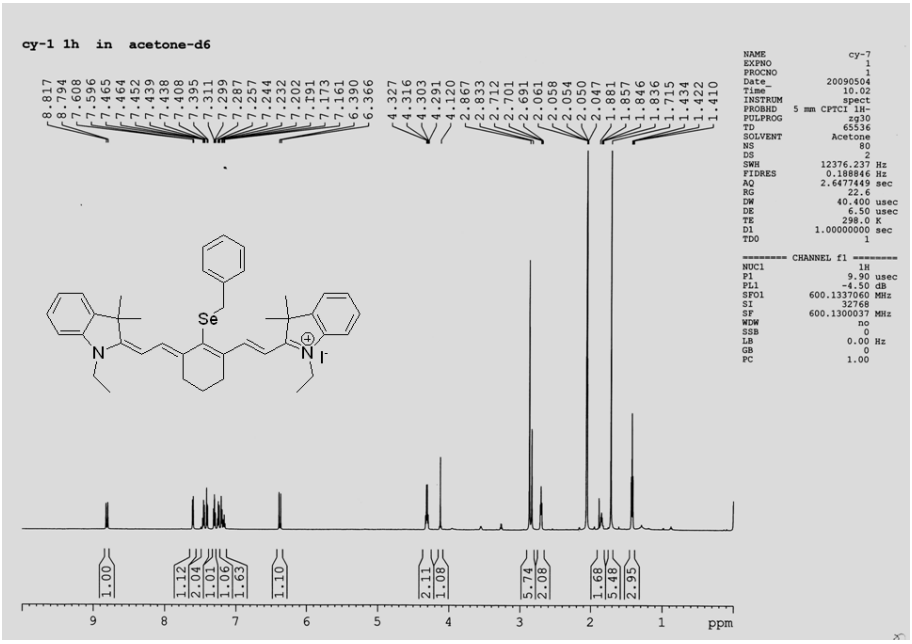



**Fig. S10.** $^{1}$H-NMR (600 MHz, Acetone-$d_6$) of BzSe-Cy.

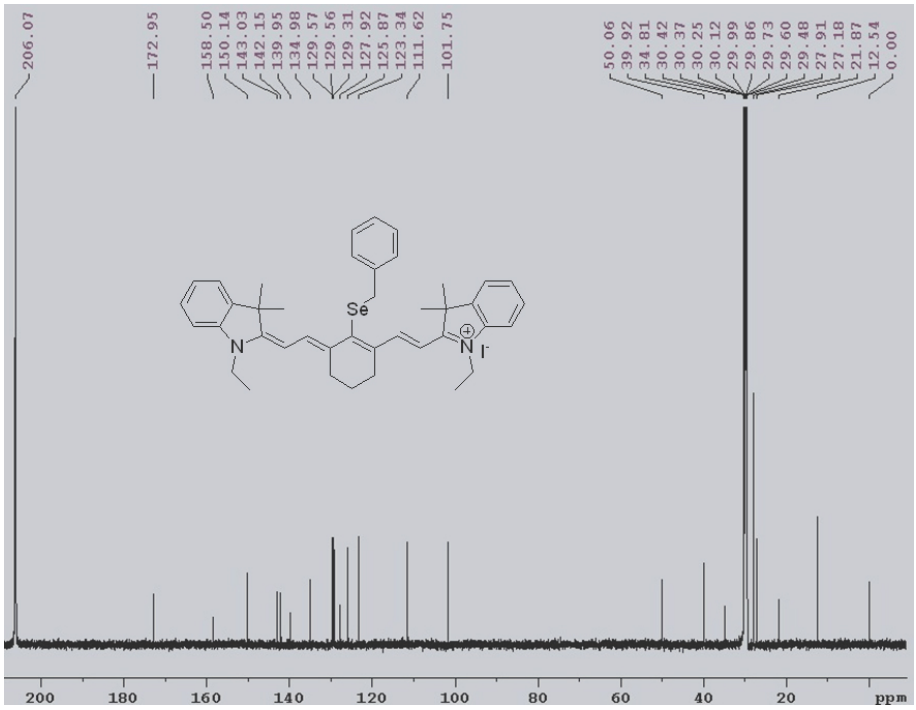



**Fig. S11.** $^{13}$C-NMR (600 MHz, Acetone-$d_6$) of BzSe-Cy.

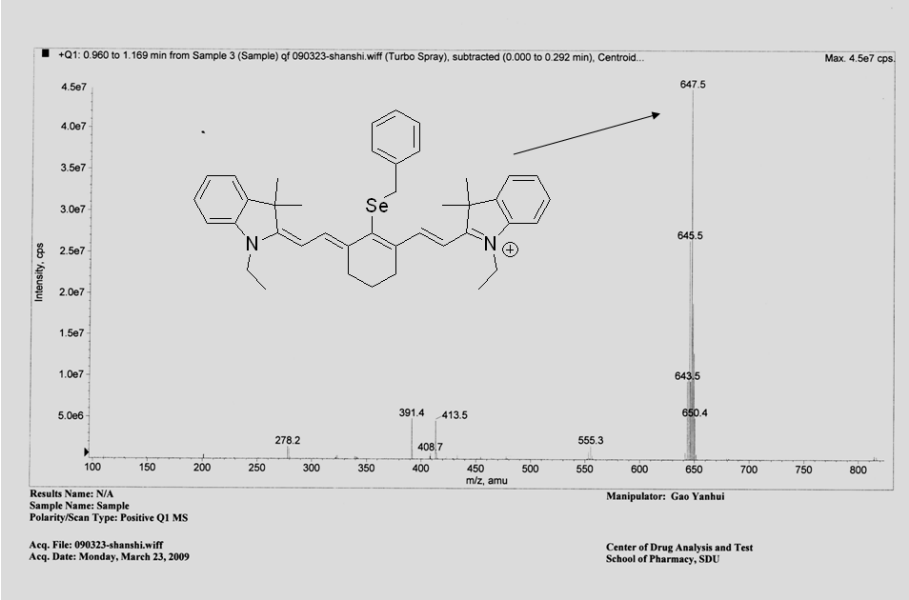



**Fig. S12.** Mass Spectrum of BzSe-Cy.

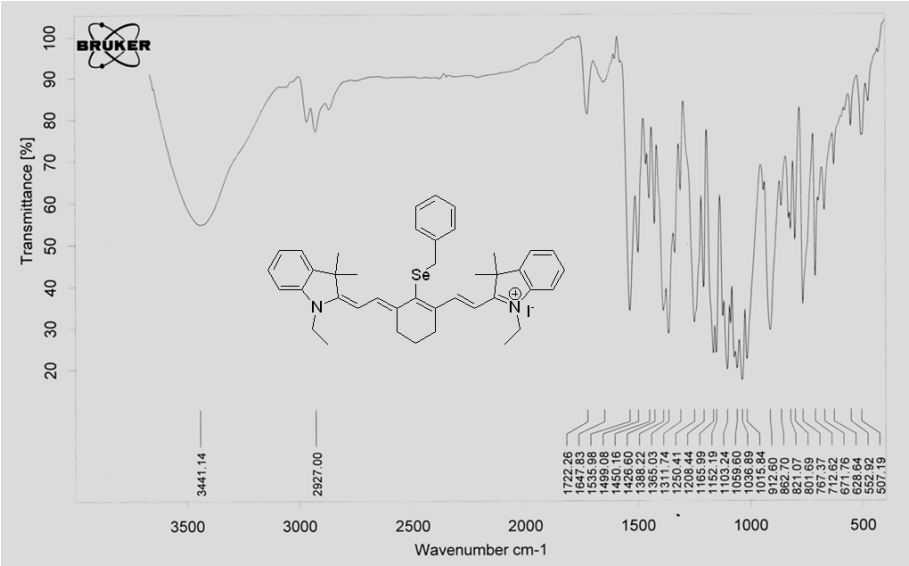



**Fig. S13.** IR spectrum of BzSe-Cy.

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