Supporting Information

A simple highly specific fluorescent probe for simultaneous discrimination of cysteine/homocysteine and glutathione/hydrogen sulfide in living cells and zebrafish using two separated fluorescence channels under single wavelength excitation

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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 biothiols in live RAW 264.7 macrophage cells and zebrafish were carried out on an Olympus FV1000-IX81 confocal fluorescence microscope. All optical measurements in this paper were carried out in PBS buffer (5 mM, pH = 7.4, containing 30% acetonitrile).

2. Determination of the detection limit

The detection limit was calculated based on the fluorescence titration. The fluorescence spectra of free probe **SNARF-NBD** were measured by five times and its standard deviation was obtained. To gain the slope, the fluorescence intensities at 643 nm were plotted as the increasing concentrations of the corresponding biothiols. 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 versus the concentrations of Cys, Hcy, GSH and H_2S.

3. Cytotoxicity assays

The cell viability of RAW 264.7 macrophage cells, treated with probe **SNARF-NBD**, 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, 5, 10, and 20 μM) of probe **SNARF-NBD** suspended in culture medium for 6 h. Subsequently, CCK-8 solution was added into each well for 2 h, and absorbance at 450 nm was measured.

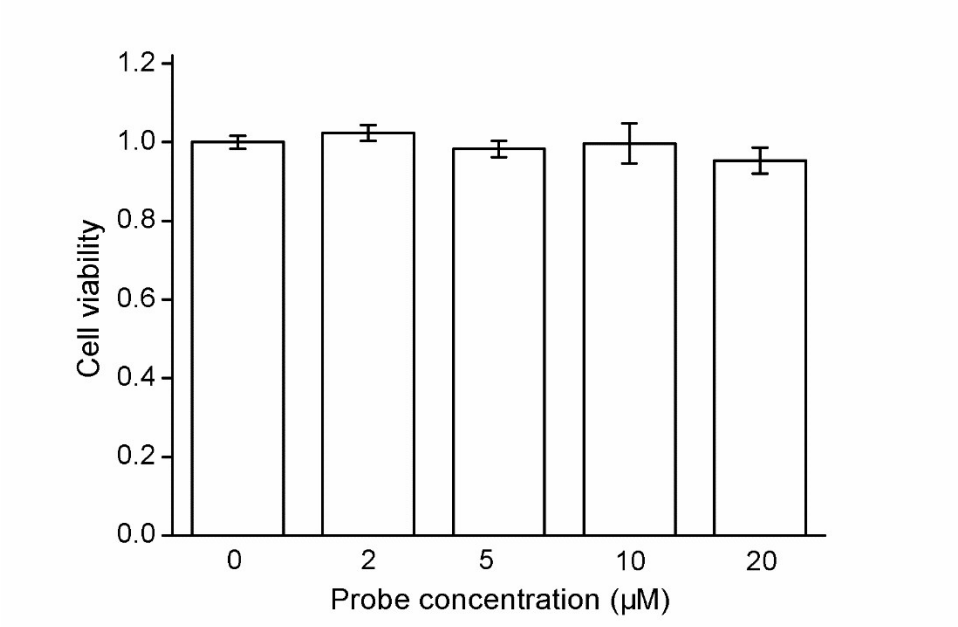

Figure S1. Cytotoxicity assays of probe **SNARF-NBD** at different concentrations for RAW 264.7 macrophage cells.

4. Imaging studies of live cells

The RAW 264.7 macrophage cells (Shanghai Institute of Biochemistry and Cell Biology, China Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) and incubated under an atmosphere containing 5% CO_2 at 37 °C humidified air for 24 h. DMEM contains 10% fetal bovine serum and 1% penicillin-streptomycin. Before imaging by confocal fluorescence microscope, probe **SNARF-NBD** (5 μM) was used as a bioimaging reagent to incubate RAW 264.7 macrophage cells for 30 min, the culture medium was then removed and rinsed with phosphate buffered saline for three times before fluorescence imaging was performed. On the other hand, the five groups of cells were treated with NEM (50 μM) for 30

minutes, washed with culture medium, and then incubated with probe **SNARF-NBD** for 30 minutes for imaging. The other four groups were treated with Cys (200 μM), Hcy (200 μM), GSH (200 μM) and H_2S (200 μM) for 30 min, then washed with culture medium and incubated with probe **SNARF-NBD** for 30 min. Then the fluorescence imaging of cells was carried out by confocal fluorescence microscope.

5. Imaging studies of zebrafish

Healthy male and female zebrafish (AB stain) were maintained in different tanks with a 14 h light / 10 h dark cycle at 28 °C. Then, sexually mature zebrafish were selected to induce spawning in tanks and the zebrafish eggs were obtained by giving light stimulation in the morning. After sterilizing and cleaning, the fertilized eggs were added to zebrafish embryo culture water (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$, 0.16 mM $MgSO_4$) and cultured in illumination incubator at 28 °C. The 6-day-old zebrafish were incubated with probe **SNARF-NBD (**5 μM) for 30 min, the culture medium was used to clean and remove the residual probe, and then confocal fluorescence microscopy was used to observe. In addition, five groups of zebrafish were treated with NEM (50 μM) for 30 minutes, one group was washed with culture medium and then incubated with probe **SNARF-NBD** for 30 minutes before imaging with confocal microscope. The other four groups were treated with Cys (200 μM), Hcy (200 μM), GSH (200 μM) and H_2S (200 μM) for 30 minutes, washed with culture medium, and then incubated with probes **SNARF-NBD** for 30 minutes. Finally, the fluorescence imaging of zebrafish was carried out by confocal fluorescence microscope.

6. Characterization of probe SNARF-NBD

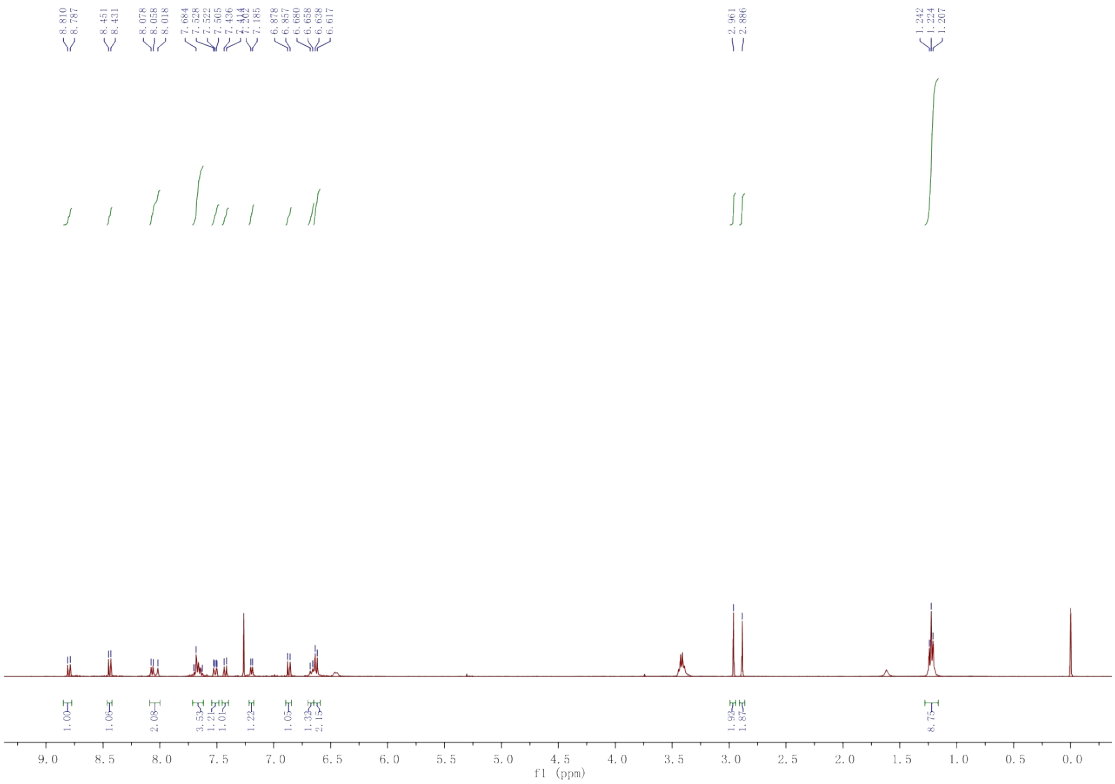

Figure S2. ^{1}H-NMR of probe **SNARF-NBD**

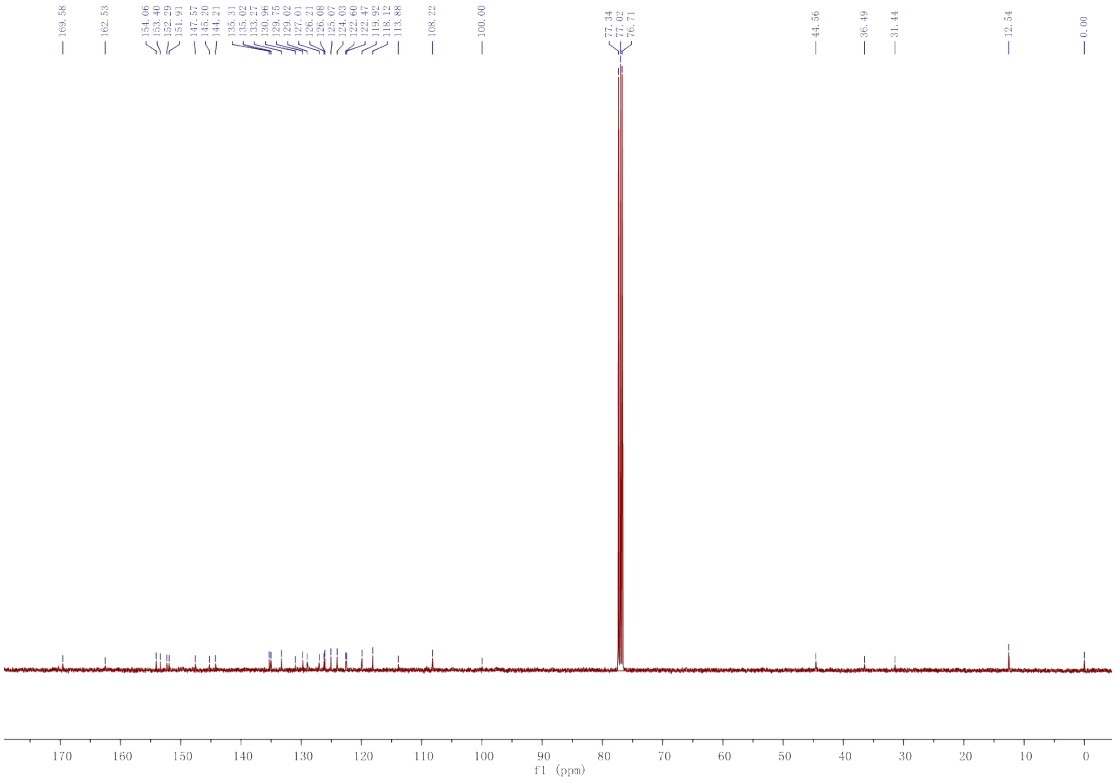

Figure S3. ^{13}C-NMR of probe **SNARF-NBD**

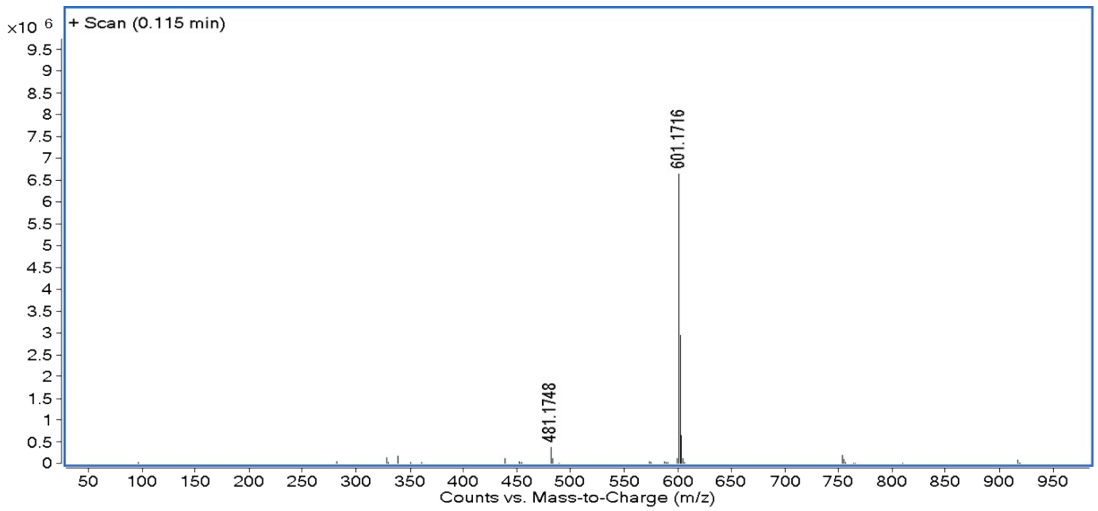

Figure S4. HRMS of probe **SNARF-NBD**