Electronic Supplementary Material (ESI) for Analyst.

Two-photon Imaging of Endoplasmic Reticulum Thiol Flux in the Brains of Mice with Depression phenotypes

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Experimental Details

Materials and instruments

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. The solvents were purified by conventional methods before use. ER-Tracker Red was purchased from Invitrogen (USA). Twice-distilled water was used throughout all experiments. N-ethylmaleimide (NEM) was purchased from Macklin. Dithiothreitol (DTT) was purchased from J&K Chemical. 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma. Silica gel (200-300 mesh) used for flash column chromatography was purchased from Qingdao Haiyang Chemical Co., Ltd. ER-SH were dissolved in dimethyl sulfoxide (DMSO) to produce 1 mM stock solutions.

^{1}HNMR and ^{13}CNMR spectra were determined by 400 MHz and 100 MHz using Bruker NMR spectrometers. The mass spectra were obtained by Bruker maxis ultra-high resolution-TOF MS system. The fluorescence spectra measurements were performed using F-4600 Hitachi fluorescence spectrometer. Cary eclipse fluorescence spectrophotometer was used for the kinetic assays. Procedures involving animals and their care were conducted in conformity with the guidelines of the Use and Care of Laboratory Animals of National Institutes of Health (NIH Pub. No. 85-23, revised 1996). All animal experiments were conducted at the laboratory of Shandong Normal University in compliance with the Guidelines of Shandong Normal University for the Care and Use of Laboratory Animals. Fluorescence imaging in cells and in vivo were performed with Leica TCS SP8 (one-photon) or Zeiss LSM 880 NLO (two-photon) Confocal Laser Scanning Microscope. The mouse mammary carcinoma cells (4T1), PC12 cells and HL7702 cells were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

Determination of the limit of detection

The detection limit was determined from the fluorescence titration data. The detection limit was calculated with the following equation: Detection limit=3σ/k, where σ is the standard deviation of blank measurement, k is the slop between the fluorescence intensity versus thiols concentration.

Fluorescence quantum yield and Measurement of Two-photon Cross Section

We choose rhodamine B as standard, which has a fluorescence quantum yield of 0.49 in ethanol.[1] Here the fluorescence quantum yield of ER-SH was 0.008 (0.01 M HEPES buffer) and the fluorescence quantum yield of ER-SH with thiols (GSH, Hcy, Cys) was 0.16, 0.27, 0.39 (0.01 M HEPES buffer), respectively. The two-photon cross section (δ) was determined by using femto second (fs) fluorescence measurement technique. ER-SH were dissolved in 0.01 M HEPES buffer, and 200 equiv thiols at the concentration of 1.0×10^{-5} M and then the two-photon induced fluorescence intensity was measured at 800 nm by using rhodamine B (1.0×10^{-5} M) as the reference, whose two-photon property has been well characterized in the literature. The intensities of the TP induced fluorescence spectra of the reference and sample emitted at the similar excitation wavelength were determined. The TPA cross section was

calculated according to Eq (1).

$$\delta_s = \delta_r \frac{\Phi_r}{\Phi_s} \frac{C_r}{C_s} \frac{n_r}{n_s} \frac{F_s}{F_r} \quad (1)$$

The subscripts s and r refer to the sample and the reference material, respectively. δ is the TPA cross sectional value, C is the concentration of the solution, n is there refractive index of the solution, F is TP excited fluorescence integral intensity and Φ is the fluorescence quantum yield. Here the fluorescence quantum yield of ER-SH was 0.39 (HEPES buffer) using rhodamine B as the reference. The result show that the TPA cross section of ER-SH in HEPES buffer at pH 7.4 is 85 GM.

Cells culture

PC12 cells, 4T1 cells and HL7702 cells were cultured in high glucose DMEM (4.5 g of glucose^{-1}) or RPMI 1640 supplemented with 10 % fetal bovine serum, 1 % penicillin, and 1 % streptomycin at 37 °C in a 5 % CO_2／95 % air incubator MCO-15AC (SANYO, Tokyo, Japan). One day before imaging, the cells were detached and were replanted on glass-bottomed dishes.

Subcellular localization fluorescence imaging in cells: The cells co-incubated with the ER-SH (20 μM) and ER-Tracker Red (500 nM) for 30 min by Leica TCS SP8 fluorescence imaging. ER-SH is green channel, λ_{ex} = 405 nm, collected at 500-550 nm. ER-Tracker Red is red channel, λ_{ex} = 561 nm, collected 590-650 nm.

Exogenous thiols imaging in live cells: The PC12 cells were divided into five groups and treated with different conditions. Control group was incubated with ER-SH (20 μM) for 30 min. Cys group and Hcy group were incubated with Cys (200 μM) and Hcy (200 μM) for 30 min, and then treated with ER-SH (20 μM) for another 30 min. NEM group was incubated with NEM (1 mM) for 30 min and then incubated with ER-SH (20 μM) for another 30 min. H_2O_2 group was incubated with H_2O_2 (200 μM) for 30 min, and then treated with ER-SH for another 30 min. Two-photon fluorescent images were acquired on LSM 880 NLO Confocal Laser Scanning Microscope with a water objective lens (×20). The excitation wavelength was 800 nm.

Endogenous thiols imaging in live cells: For Fig. S8, the cells were incubated with ER-SH (20 μM) for 30 minutes, and then incubated with DTT (5 mM) over time for imaging. For Fig. 5, PC12 cells were divided into three groups and treated with different conditions. The control group was incubated with ER-SH (20 μM) for 30 min. The second group was pre-treated with DTT (5 mM) for 60 min, and then incubated with ER-SH (20 μM) for another 30 min. The third group was pre-treated with DTT (5 mM) for 120 min, and then incubated with ER-SH (20 μM) for another 30 min. TP fluorescent images were acquired on LSM 880 NLO Confocal Laser Scanning Microscope with a water objective lens (×20). The excitation wavelength was 800 nm.

Cytotoxicity assays

The cytotoxicity of ER-SH towards 4T1 cell lines was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 4T1 cells were seeded in a 96-well plate at a concentration of 1×10^5 cells well^{-1} in 100 μL of Roswell Park Memorial Institute (RPMI-1640) medium with 10 % fetal bovine serum, 1 % penicillin, and 1 % streptomycin and maintained at 37 °C in a 5% CO2 incubator for 12 hours. Then, cells were exposed to different concentrations of ER-SH, (0.1, 1, 10, 100 μM) for 24

hours. The total volume of 96-well microtiter plates is 200 μL well^{-1}. The cells were washed with 37 °C HEPES and MTT solution (5 mg mL^{-1}, 20 μL) was added to each well and continuously incubated for 4 h at 37 °C. After 4 h, MTT solution was removed and DMSO (150 μL) was added to each well to dissolve the dark blue formazan crystals. Absorbance was measured at 490 nm in a Triturus microplate reader (Fig. S5). Data indicated that ER-SH has no marked cytotoxicity at concentrations below 0.20 mM.[2]

Fluorescence imaging in zebrafish

Zebrafish were divided into three groups and treated with different conditions. The first group was incubated with ER-SH (20 μM) at different times for 0.5 h, 1 h, 2 h, 12 h, 24 h, 48 h. The second group was incubated only with the probe of ER-SH (20 μM) for 1 h. Fluorescence images of the zebrafish were incubated with NEM (1 mM) and then incubated with ER-SH (20 μM) for another 1 h. The three group was incubated with ER-SH (20 μM) for 1 h, and then zebrafish transferred to normal medium and incubated to observe the change of fluorescence intensity with different times. TP fluorescent images were acquired on LSM 880 NLO Confocal Laser Scanning Microscope with an air objective lens (×10). The excitation wavelength was 800 nm.

Mouse models

Mice

All female KM mice and adult male C57BL/6J mice (age: 6 weeks; average body weight: 18 ± 2 g) were purchased from Shandong University Laboratory Animal Center. Mice were housed singly in cages under a controlled 12-hour/12-hour light-dark cycle (lights on: 7:00 a.m.). Mice were given free access to water and food. Mice were allowed to adapt to the stable environmental conditions for 1 week. All the animal experiments were carried out in accordance with the relevant laws and guidelines issued by the Ethical Committee of Shandong University.

DTT-induced Mice and NEM-induced Mice:

All female KM mice were divided into three groups and treated with different conditions. Blank mice: the mice were pre-treated with HEPES for 4 hours and then injected ER-SH (100 μM) for 15 minutes. DTT-induced mice: the mice were pre-treated with DTT (10 mM) for 4 hours and injected ER-SH for 15 minutes. The mice were pre-treated with NEM (1 mM) for 4 hours, then the mice were injected ER-SH for 15 minutes. Then, mice were anesthetized with 4 % chloral hydrate (3 mL kg^{-1}) by intraperitoneal injection (i.p.) and the hair and skin on the ventral surface of the abdomen was removed. Two-photon fluorescent images were acquired on a LSM 880 NLO Confocal Laser Scanning Microscope with a water objective lens (×20). The excitation wavelength was 800 nm.

Mouse Models of Depression

Mouse models of depression were constructed by chronic unpredictable mild stress (CUMS) and separation according to references.[3] The mice were received a variety of disposed independently and randomly, including soiled cage, empty cage with water on the bottom, continuous overnight illumination, inversion of the light/dark cycle for 28 days.

Results and Discussion:

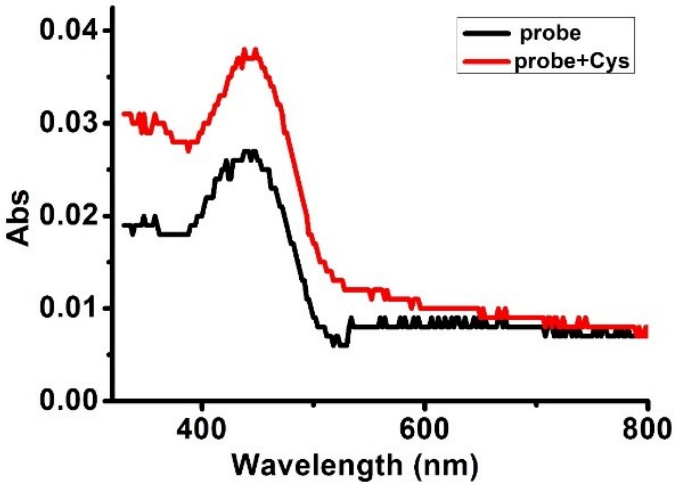

Figure S1. Absorption spectra of 10 μM ER-SH with Cys (100 equiv). The Black line is ER-SH. The red line is absorbed in the presence of Cys.

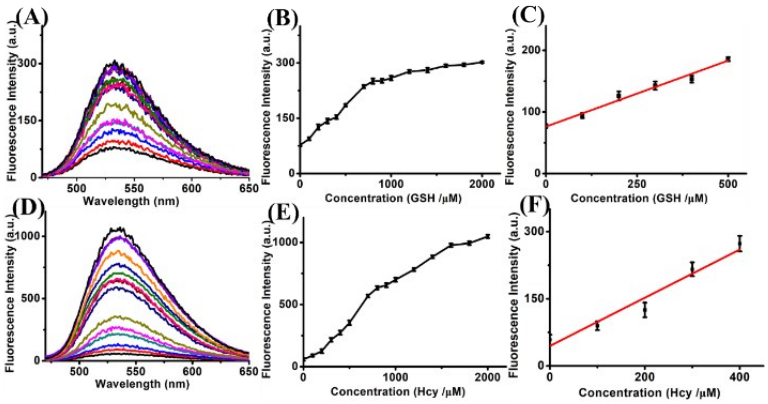

Figure S2. Fluorescence responses of ER-SH with different concentrations of GSH and Hcy. (A, B) and (D, E) were the fluorescence intensity changes of 10 μM ER-SH with different concentrations of GSH and Hcy (0-200 equiv), respectively. (C) and (F) were the linear of ER-SH with different concentrations of GSH (0-500 μM) and Hcy (0-400 μM), respectively. Excitation wavelength at 420 nm and emission wavelength at 535 nm. Error bars are ± SD (n=5).

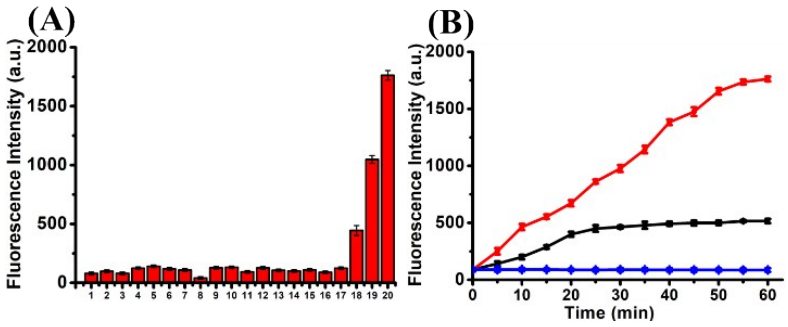

Figure S3. Fluorescence responses of ER-SH with different conditions. (A) Fluorescence responses of ER-SH (10 μM) reacted with various competed species for 50 min. 1-20: blank, Na^+ (10 mM), K^+ (10 mM), Mn^{2+} (100 μM), Cu^{2+} (100 μM), Al^{3+} (100 μM), Fe^{3+} (100 μM), Mg^{2+} (0.5 mM), Zn^{2+} (0.5 mM), Ca^{2+} (0.5 mM), citric acid (100 μM), $Na_2S_2O_3$ (100 μM), H_2O_2 (10 mM), $^1O_2$ (100 μM), NO (100 μM), TBHP (100 μM), $ONOO^-$ (100 μM), GSH (2 mM), Hcy (2 mM), Cys (2 mM). (B) The blue line is the fluorescence change of ER-SH (10 μM) with time. The black line is fluorescence change of ER-SH (10 μM) over time when Cys (2 mM) is mixed with H_2O_2 (2 mM). The red line is fluorescence responses of ER-SH to Cys (2 mM) at different time. It can be seen from the figure that Cys will undergo slow oxidation in the presence of H_2O_2. Excitation wavelength at 420 nm and emission wavelength at 535 nm. Error bars are ± SD (n=5).

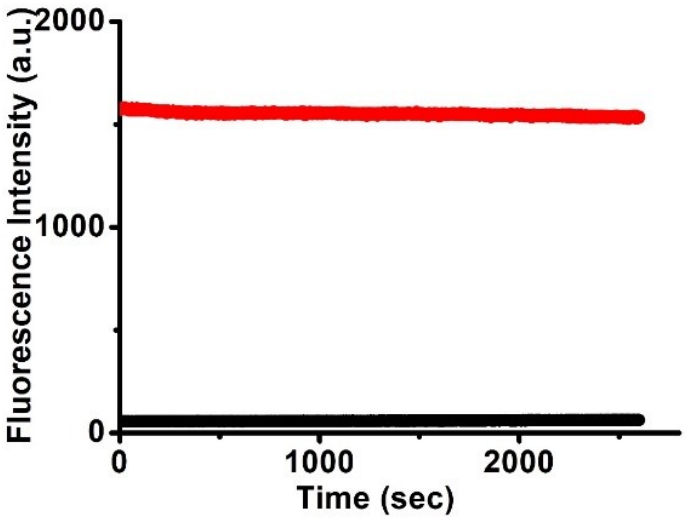

Figure S4. Photostability experiments of 10 μM ER-SH to 200 equiv Cys. The Black line is ER-SH. The red line is fluorescence intensity in the presence of Cys. Excitation wavelength at 420 nm and emission wavelength at 535 nm.

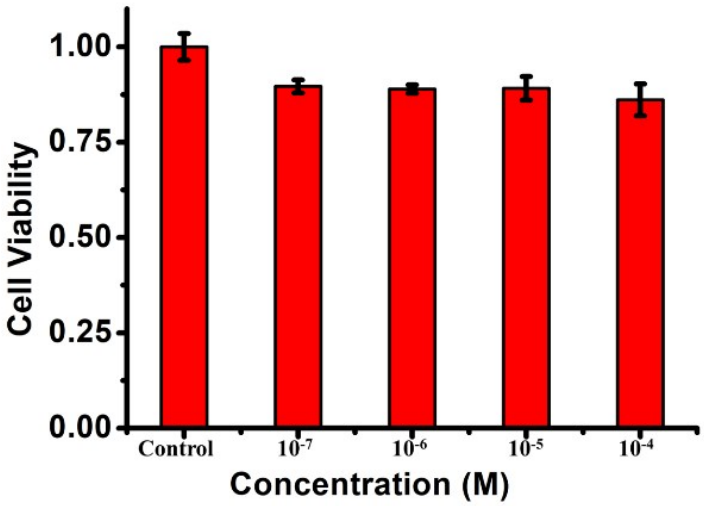

Figure S5. Cytotoxicity assay of ER-SH.

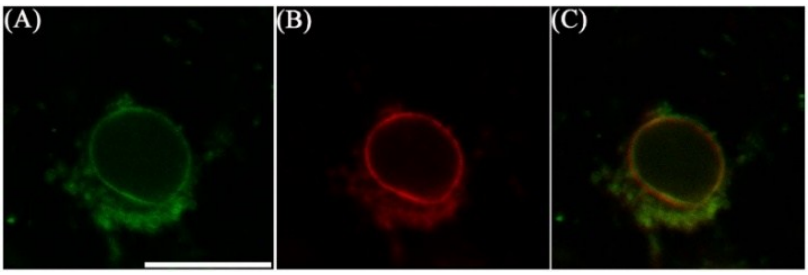

Figure S6. Co-localization imaging of ER-SH (20 μM) with commercial ER-Tracker Red (500 nM) dyes in 4T1 cells. (A) ER-SH is green channel, λ_{ex} = 405 nm, collected at 500-550 nm. (B) ER-Tracker Red is red channel, λ_{ex} = 561 nm, collected 590-650 nm. (C) Merged image of A and B. Scale bar: 25 μm.

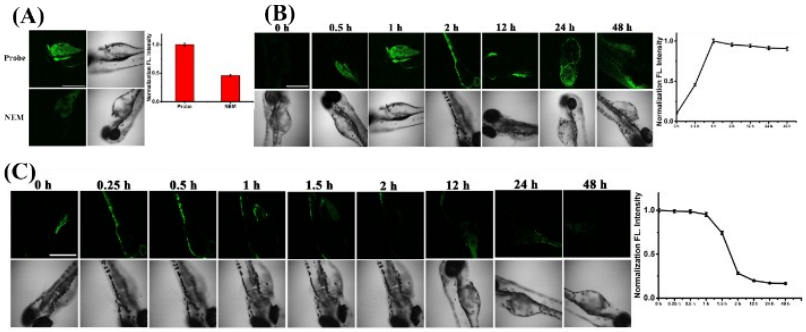

Figure S7. TP imaging in zebrafish. (A) The first line was the zebrafish incubated only with the probe of ER-SH (20 μM) for 1 h. The second line was fluorescence images of the zebrafish incubated with NEM and then incubated with ER-SH for another 1 h. The second series contain bright-field images. Histogram: Normalization fluorescence intensities of images of zebrafish. (B) TP fluorescence images of zebrafish were stained ER-SH (20 μM) at different times. The second line contain bright-field images. Normalization fluorescence intensities of images of zebrafish. (C) After being incubated with ER-SH (20 μM) for 1 h, zebrafish were transferred to normal medium and incubated to observe the change of fluorescence intensity with different times. The second line contain bright-field images. Normalization fluorescence intensities of images of zebrafish. Two-photon excitation wavelength was at 800 nm and emission windows: 500-600 nm. Scale bar: 50 μm.

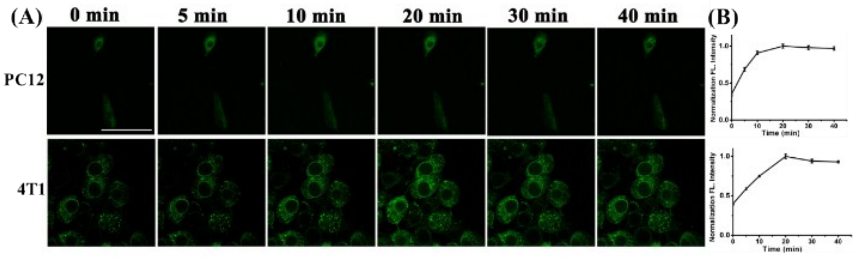

Figure S8. TP imaging of cells with DTT over time. (A) PC12 cells were firstly incubated with ER-SH (20 μM) for 30 minutes, and then treated with DTT (5 mM). TP imaging of PC12 cells and 4T1 cells in the presence of DTT at different times. (B) Normalization fluorescence intensities of PC12 cells and 4T1 cells in image A at different times, respectively. Two-photon excitation wavelength was at 800 nm and emission windows: 500-600 nm. Scale bar: 20 μm.

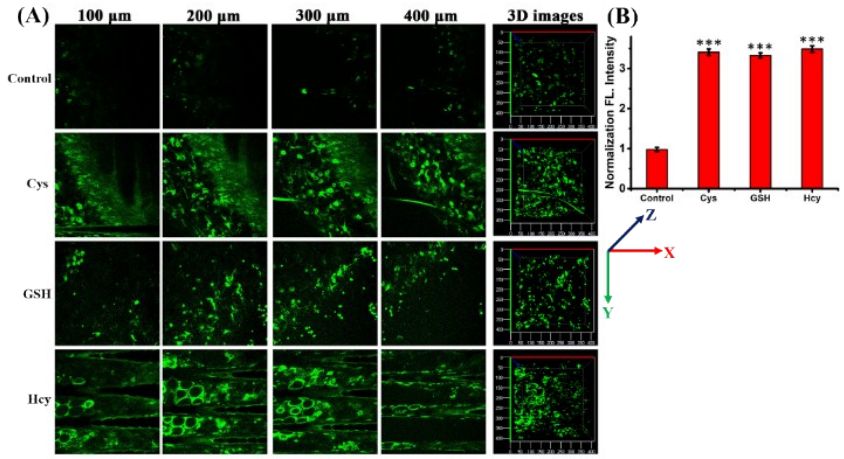

Figure S9. TP imaging of exogenous thiols in abdomen of mice. (A) The fluorescence images of tissue was stained with ER-SH (100 μM) at different depths and the 3D stack images. Control (n=5). The mice were given an i.p. injection of Cys (n=5), Hcy (n=5), and GSH (n=5) (100 μM) followed by an i.p. injection with ER-SH, respectively. (B) Normalization fluorescence intensity statistics were output by selecting five representative regions. Relative fluorescence intensities of 3D images of living tissue in (A). Data are expressed as the mean ± S.D., ***P< 0.001 for Cys, Hcy, and GSH vs. control. The two-photon laser was 800 nm, and fluorescence was collected from 500-600 nm. Scale bar =50 μm.

References

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3 N. Dedic, S. M. Walser and J. M. Deussing, *Psychiatric Disorders-Trends and Developments*, 2011, 185-222.