## Supporting Information

# A coumarin based reversible two-photon fluorescence probe for imaging glutathione near N-methyl-D-aspartate (NMDA) receptors

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#### 1. Materials and general methods.

All chemicals were obtained from commercial suppliers and used as received. <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded using Bruker 300MHz or Varian 500MHz. UV absorption spectra were obtained on Evolution 201 (Thermo Scientific). Fluorescence emission spectra were recorded using RF-5301/PC (Shimadzu) spectrophotometer. All the spectroscopic experiments were performed in a 1cm X 1cm quartz cuvette. All experiments were approved by the Institutional Animal Care and Use Committee of Ajou University (IACUC).

## 2. Synthesis



Scheme S1. Synthetic route for probe probe 1.

Compound 2,3 and 4 were prepared according to the literature reported work. S1-S3

#### (E)-N-(6-((2-(4-benzylpiperidin-1-yl)-1-(4-hydroxyphenyl)propyl)amino)hexyl)-2-cyano-3-(7-

(diethylamino)-2-oxo-2H-chromen-3-yl)acrylamide (1) (E)-2-cyano-3-(7-(diethylamino)-2-oxo-2H-chromen-3-yl)acrylic acid (221 mg, 0.71 mmol, 1 eq.) was dissolved in anhydrous DMF (5 ml) under N2 condition. After cooling to 0 °C, HOBt (191 mg, 1.42 mmol, 2 eq.) was added to the solution and stirred for 10 min. 4-(1-((6aminohexyl)amino)-2-(4-benzylpiperidin-1-yl)propyl)phenol (300 mg, 0.71 mmol, 1 eq.) was added to the solution and 10 min later, TEA, 0.4 mL, 2.8 mmol, 4 eq. was added dropwise, 15 min later, EDC (271 mg, 1.42 mmol, 1 eq.) was added into the mixture dropwise. The reaction mixture was stirred at room temperature for 24 h. After the solvent was evaporated under reduced pressure, the residue was purified by silica gel column chromatography using MC/MeOH (95:5, v/v) to afford (E)-N-(6-((2-(4-benzylpiperidin-1-yl)-1-(4hydroxyphenyl)propyl)amino)hexyl)-2-cyano-3-(7-(diethylamino)-2-oxo-2H-chromen-3-yl)acrylamide as red colored solid. Yield. 136 mg, (26.8%). <sup>1</sup>H NMR (300 MHz, CHLOROFORM-D) & 8.66 (s, 1H), 8.49 (s, 1H), 7.39-7.34 (m, 3H), 7.28-7.23 (m, 3H), 7.19-7.10 (m, 3H), 7.00-6.98 (d, J=7.28 Hz, 2H), 6.98-6.60 (dd, J=8.46, 1.64 Hz, 1H), 6.56-6.52 (t, 5.05, 3.99 Hz, 1H), 6.45-6.44 (d, J=2.23 Hz, 1H), 3.61-3.57 (d, J=10.69 Hz, 1H), 3.50-3.34 (m, 6H), 3.14-3.11 (d,10.10 Hz, 1H), 2.86-2.74 (m, 2H), 2.54-2.52 (d, J=6.81 Hz, 4H), 2.28-2.20 (m, 1H), 1.81-1.73 (m, 2H), 1.63-1.52 (m, 4H), 1.29-1.21 (m, 14H), 0.76-0.74 (d, 7.28 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CHLOROFORM-D) & 161.01, 160.84, 157.80, 153.21, 145.66, 143.70, 143.60, 140.51, 131.72, 130.12, 129.16, 128.28, 127.76, 125.92, 124.18, 123.81, 118.53, 117.77, 116.53, 111.52, 111.43, 110.21, 108.59, 101.61, 97.23,

97.19, 64.42, 61.98, 45.35, 45.02, 44.85, 43.04, 40.14, 38.13, 32.54, 29.78, 29.16, 26.85, 26.26, 26.14, 12.58, 9.26. HRMS (ESI) calcd for  $C_{44}H_{56}N_5O_4$  [M+H]<sup>+</sup> 718.4288, found 718.4322.

**3. Spectroscopic Measurements** 



**Figure S1**. Fluorescent changes of probe 1 (10  $\mu$ M) and GSH (5 mM) followed by the addition of H<sub>2</sub>O<sub>2</sub> (10 mM) in 10 mM PBS buffer (pH 7.4, containing 30 % DMF,  $\lambda_{ex}$  = 500 nm, slit: 1.5/3 nm).



**Figure S2**. Response time (left) and dissociation constant (Kd) (right) of probe 1. Time-course fluorescence response spectra of 1 (10  $\mu$ M) towards GSH (10 mM) in PBS buffer (pH 7.4, 10 mM, containing 30 % DMF,  $\lambda_{ex} = 400$  nm, slit: 1.5/3 nm) inset describes the observed rate constant (K<sub>obs</sub>), calculated half-time (t<sub>1/2</sub>) and rate constant (K). Fluorescent intensity ratio (I<sub>475</sub>nm/I<sub>560</sub>nm) changes of 1 (10  $\mu$ M) as a function of the concentration of GSH (0 - 10 mM) in PBS buffer (pH 7.4, 10 mM, containing 30% DMF). Each spectrum was recorded after 30 min. The reciprocal of the slope is the dissociation constant K<sub>d</sub> (based on fluorescent intensity ratio measurements).



**Figure S3.** Time-course fluorescence changes of **1** (10 uM) upon addition of GSH (10 mM) in the absence/presence of cellular interfering thiols (Cys: 200 $\mu$ M, Hcy: 10uM) (PBS pH 7.4, 10 mM, containing 30 % DMF,  $\lambda_{ex} = 400$  nm,  $\lambda_{em} = 475$ nm, slit: 1.5/3nm).



**Figure S4**. Fluorescence spectra of **1** (10  $\mu$ M) at varied pH values (left:  $\lambda_{ex} = 400$  nm, right :  $\lambda_{ex} = 500$  nm, slit : 1.5/3nm).



**Figure S5.** (a) Fluorescence spectra of Probe **1** (10 $\mu$ M)+GSH (5mM) in PBS (30% DMF, 1% DMSO) at varied pH values ( $\lambda_{ex} = 400$  nm, slit : 2.5/2.5nm), (b) Fluorescence spectra of Probe **1** (10 $\mu$ M)+GSH (5mM)+H<sub>2</sub>O<sub>2</sub> (10mM) in PBS (30% DMF, 1% DMSO) at varied pH values ( $\lambda_{ex} = 500$  nm, slit : 2.5/2.5nm),.



**Figure S6**. (left) Fluorescence intensity of probe 1 towards various intracellular weak nucleophiles (Thr, Ser, Lys, Trp, Arg, His), histamine, imidazole, and reductants (Vitamin C and NADH) (1 mM). (right) Fluorescence spectra of 1 (10 $\mu$ M) in PBS buffer to various ROS. 1 mM for ClO<sup>-</sup>, <sup>1</sup>O<sub>2</sub>, H<sub>2</sub>O<sub>2</sub> and 0.1 mM for ·OH (pH 7.4, 10 mM, containing 30% DMF) ( $\lambda_{ex}$  = 400 nm; Slits: 1.5 / 3 nm).



**Figure S7**. Reversibility cycles of probe 1 (10  $\mu$ M) with 5mM GSH and 10mM H<sub>2</sub>O<sub>2</sub> ( $\lambda_{ex} = 400$  nm,  $\lambda_{em} = 475$  nm, PBS pH 7.4, 10 mM, containing 30 % DMF)

### 4. Cell Culture

HeLa cells were grown in Minimum Essential media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and kept in an incubator at 37 °C under a 5% CO<sub>2</sub> atmosphere. Primary cortical neurons were prepared from 1-day-old wild-type (WT) pups and transgenic mice. After transfer to glass bottomed dishes (NEST), and incubated in a Neurobasal medium (Invitrogen, Carlsbad, CA) with B-27 supplement and L-glutamine (Invitrogen, Carlsbad, CA) for 2 weeks before imaging. Cells were maintained in 5% CO<sub>2</sub> at 37 °C. For staining, growth medium was removed and change to serum-free medium. The cells were incubated with 10  $\mu$ M probe 1 at 37 °C under 5% CO<sub>2</sub> for 30 min. After incubation, they were washed three times with phosphate buffered saline (PBS, Gibco) and imaged in colorless serum-free medium.

#### 5. Cell viability test

HeLa cells were seeded in a 96-well plate at a density of  $3x10^4$  cells per well in culture media at 37°C and 5% CO<sub>2</sub> and incubated for 24h. After incubation, the cells were treated with different concentrations of probe 1 for

24h. After washing, cells were cultured in 100  $\mu$ L of fresh culture medium and 20  $\mu$ L of MTT reagent for 4h at 37 °C. The cell viability was monitored by measuring the absorption at 650nm using a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices).



Figure S8. Cell viability after 24h of culture with different concentrations of probe 1.

#### 6. Measurement of the two-photon cross section

The two-photon action cross section ( $\delta\Phi$ ) was measured using Rhodamine 6G as the reference. Probe 1 was dissolved in PBS buffer (10 mM, pH = 7.4) at concentrations of 10 µM and then the two-photon excited fluorescence intensity value was measured at 720–960 nm. To measure the two-photon cross-sectional area ( $\delta$ ) of the reaction product between probe 1 (10 µM) and GSH (500 µM), it was stored at room temperature for about 1 hour. The TPA cross section was calculated using the equation  $\delta = \delta r (Ss \Phi r \varphi r cr)/(Sr \Phi s \varphi s cs)$ . In the equation, s and r denote the sample and reference molecules, respectively. The fluorescence intensity measured by the CCD detector corresponds to S. The quantum yield is expressed as  $\Phi$ .  $\varphi$  indicates the overall fluorescence collection efficiency of the experimental devices. The concentration of the object to be measured was indicated by c.  $\delta r$  corresponds to the TPA cross section value of the reference (Rho6G).



**Figure S9**. Two-photon action spectra of the 10  $\mu$ M probe 1 in the absence and presence of 500  $\mu$ M GSH in PBS Buffer (10 mM, pH = 7.4).

#### 7. Two-photon fluorescence microscopy

Two-photon excited fluorescence microscopy images were obtained after staining cells and tissues with probe 1 using multiphoton microscopes (Leica TCS SP8 MP) with a ×40 oil, numerical aperture (NA = 1.30) objective lens. Two-photon fluorescence microscopy images were obtained with a DMI6000B Microscope (Leica) compatible with a mode-locked titanium-sapphire laser source (Mai Tai HP; 80 MHz pulse frequency, Spectra Physics, 100 fs pulse width), and probe was excited at a 750 nm wavelength with an output power of 2670 mW, which corresponding to average power of approximately 2.5 mW at the focal plane. To obtain TPM images at 450-600 nm range, internal PMTs were used to acquire the signals in 8-bit unsigned  $512 \times 512$  pixels at a 400 Hz



scan speed.

**Figure S10**. (a) TPM image of Probe-labeled (10  $\mu$ M) primary cortical neuronal cells collected at 450-650 nm. (b) The relative TPEF intensity as a function of time. The digitized intensity was recorded with 2.0 sec intervals for the duration of one hour using *xyt* mode ( $\lambda_{ex} = 750$  nm, ~200 fs). Cells shown are representative images from replicate experiments (n = 5). Scale bar, 60  $\mu$ m.

### 8. Preparation and staining of fresh rat Hippocampal slices

Rat Hippocampal slices for imaging were extracted from the hippocampus of two week old rats (SD). Coronal slices were cut into 400  $\mu$ m-thick samples using a vibrating-blade microtome with artificial cerebrospinal fluid (ACSF; 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM D-glucose, 124 mM NaCl, 3 mM KCl, 2.4 mM CaCl<sub>2</sub>, 26 mM NaHCO<sub>3</sub>, and 1.3 mM MgSO<sub>4</sub>). The prepared slices were incubated with 30  $\mu$ M probe 1 for 1 hour in ACSF bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37 °C, then after washed three times with ACSF. Slices were transferred to a glass-bottomed dish (MatTek) for imaging. To confirm the possibility of GSH level change measurements, slices were pretreated with 300  $\mu$ M NEM for 50 minutes prior to probe 1 treatment.

## 9. NMR and mass spectrum



Figure S11. <sup>1</sup>H NMR spectrum (300MHz) of probe 1.



Figure S12. <sup>13</sup>C NMR spectrum (75MHz) of probe 1.



Figure S13. The ESI-mass spectrum of probe 1. Probe 1  $[M+H]^+$  signal is observed at m/z 718.4344 (expected m/z 718.4288  $[M+H]^+$ ).

#### **10. Reference**

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