Supporting Information for

Fluorescent probes guided by new practical performance regulation strategy to monitor glutathione in living systems

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1. Experimental procedures and characterisation data

1.1 Materials

The absorbance spectra were measured on a Shimadzu UV-1700 spectrophotometer. Fluorescent spectra data were recorded by a Hitachi F-4500 fluorescence spectrophotometer equipped with a xenon discharge lamp and 1 cm quartz cell. NMR spectra were collected on a Bruker-AVANCE III 400 MHz spectrometer (at 400 MHz for $^1$H and 100 MHz for $^{13}$C) using tetramethylsilane (TMS) as the internal standard. Mass spectra were performed with Bruker micrOTOF-Q II ESI-Q-TOF LC/MS/MS Spectroscopy. Bioimaging of the sensors was performed on an Olympus FV1000 confocal microscope and the excitation wavelength was set at 488 nm. Flow cytometric analysis was carried out on BD Biosciences AccuriC6 and live mouse imaging was performed on PerkinElmer Lumina LT Series III. Isoflurane was used for anesthesia. All the live subjects procedures were conducted in accordance with the Experimental Animal Administration regulations issued by the State Committee of Science and Technology of the People's Republic of China and Experiments were approved by the Animal Ethics Committee of Northwest University.

Fluorescein, cinnamic acid derivatives, amino acids, α-lipoic acid, and N-Ethylmaleimide were obtained from TCI (Shanghai) Development Co., Ltd. Analytical thin layer chromatography was performed using Merck 60 GF254 silica gel (pre-coated sheets, 0.25 mm thick). Silica gel (0.200-0.300 mm, 60 A, J&K Scientific Ltd.) was used for column chromatography.

1.2 Synthesis and characterization of all probes

Fluorescein was acidize with 4 mol/L hydrochloric acid, and dry it in a vacuum oven at 60°C for 4 hours for subsequent synthesis. 0.1 mmol cinnamic acid derivatives were acylated by thionyl chloride (0.2 mmol) in 50 ml dichloromethane at 0 °C and stirred for 5 h, remove the solvent and thionyl chloride by vacuum, and add 0.2 mmol triethylamine and stirred for 0.5 h, then the aforementioned acidize fluorescein (0.1 mmol) were suspended in 20 ml dichloromethane and dropped into to the solution in 1 h. The mixture were stirred for another 6 h at 0 °C, then remove the solvent, and purified by column chromatography using dichloromethane : methanol (v/v) = 30:1 as eluent.
The characterization data were shown as followed:

**FI-H:** white powder, yield: 83.35 %.

1H NMR (400 MHz, d6-DMSO, TMS) δ 10.26 (s, 1H), 8.06 (d, J = 7.6 Hz, 1H), 7.92 (d, J = 16.0 Hz, 1H), 7.85 (t, J = 5.8 Hz, 3H), 7.77 (t, J = 7.4 Hz, 1H), 7.49 (d, J = 4.4 Hz, 3H), 7.39 (d, J = 7.6 Hz, 1H), 7.37 (s, 1H), 7.02 (d, J = 8.4 Hz, 1H), 6.94 (d, J = 16.0 Hz, 1H), 6.87 (d, J = 8.8 Hz, 1H), 6.75 (s, 1H), 6.63 (s, 2H). 13C NMR (100 MHz, d6-DMSO, TMS) δ 169.0, 165.0, 160.2, 152.8, 152.3, 152.0, 151.7, 147.5, 136.3, 134.2, 131.5, 130.8, 129.6, 129.5, 129.2, 126.3, 125.3, 124.6, 118.6, 117.2, 117.1, 113.6, 110.9, 109.6, 102.8, 82.5, 55.4. EMS/MS m/z calcd. for C29H17O6^- ([M-H]^-): 461.1031, found 461.1035.

**FI-α-Me:** flaxen powder, yield: 79.41 %.

1H NMR (400 MHz, (CD3)2CO, TMS) 8.03 (d, J = 7.6 Hz, 1H), 7.05 (s, 1H), 7.85 (t, J = 7.6 Hz, 1H), 7.78 (t, J = 7.6 Hz, 1H), 7.59 (d, J = 7.6 Hz, 2H), 7.50 (d, J = 7.6 Hz, 2H), 7.44 (d, J = 7.6 Hz, 1H), 7.38 (d, J = 7.6 Hz, 1H), 7.30 (s, 1H), 7.03 (d, J = 8.4 Hz, 1H), 6.94 (d, J = 8.4 Hz, 1H), 6.81 (s, 1H), 6.72 (q, J = 8.4 Hz, 2H), 2.24 (s, 3H). 13C NMR (100 MHz, (CD3)2CO, TMS) δ 168.49, 166.14, 159.56, 152.96, 152.79, 152.20, 151.76, 140.64, 138.39, 135.44, 135.39, 130.10, 129.90, 129.64, 129.33, 129.06, 128.92, 128.57, 128.44, 127.37, 126.63, 124.67, 124.09, 117.98, 117.07, 112.80, 110.38, 102.49, 81.94, 13.57. EMS/MS m/z calcd. for C30H19O6^- ([M-H]^-): 475.1187, found 475.1311

**FI-β-Me:** flaxen powder, yield: 88.47 %.

1H NMR (100 MHz, CDCl3, TMS) 8.02 (d, J = 6.8 Hz, 1H), 7.63 (quint, J = 5.6 Hz, 2H), 7.55 (d, J = 3.6 Hz, 2H), 7.43 (d, J = 3.2 Hz, 2H), 7.14 (s, 1H), 7.07 (d, J = 7.6 Hz, 1H), 6.82 (q, J = 8.4 Hz, 2H), 6.8 (s, 1H), 6.62 (d, J = 8.8 Hz, 1H), 6.52 (d, J = 8.8 Hz, 1H), 6.38 (s, 1H), 6.28 (s, 1H), 2.65 (s, 3H). 13C NMR (100 MHz, CDCl3, TMS) δ 170.2, 165.1, 160.2, 158.6, 153.1, 152.1, 152.0, 151.9, 141.6, 135.3, 129.9, 129.7,
129.1, 129.1, 128.7, 126.5, 126.4, 125.1, 124.1, 117.6, 116.4, 115.4, 112.8, 110.6, 110.1, 103.1, 83.4, 18.5. EMS/MS m/z calcd. for C$_{30}$H$_{19}$O$_6$ ([M-H]$^-$): 475.1187, found 475.1162.

Fl-2OMe: flaxen powder, yield: 85.97 %.

$^1$H NMR (400 MHz, CDCl$_3$, TMS) δ 8.19 (d, $J = 16.4$ Hz, 1H), 8.02 (d, $J = 7.2$ Hz, 1H), 7.63 (quint, $J = 6.8$ Hz, 2H), 7.57 (d, $J = 7.6$ Hz, 1H), 7.41 (t, $J = 7.6$ Hz, 1H), 7.16 (s, 1H), 7.08 (d, $J = 7.2$ Hz, 1H), 7.00 (t, $J = 7.6$ Hz, 1H), 6.96 (d, $J = 8.0$ Hz, 1H), 6.83 (dd, $J_1 = 14.0$ Hz, $J_2 = 8.8$ Hz, 2H), 6.74 (d, $J = 16.4$ Hz, 1H), 6.69 (s, 1H), 6.63 (d, $J = 8.8$ Hz, 1H), 6.53 (d, $J = 8.4$ Hz, 1H), 6.27 (br, 1H), 3.93 (s, 3H).

$^{13}$C NMR (100 MHz, (CD$_3$)$_2$CO, TMS) δ 168.5, 164.8, 159.6, 158.7, 152.9, 152.5, 152.2, 151.7, 142.0, 135.4, 132.5, 130.1, 129.3, 129.2, 129.0, 126.6, 124.7, 124.1, 122.6, 120.8, 117.9, 117.0, 116.9, 112.8, 111.6, 110.3, 102.6, 82.0, 55.2. EMS/MS m/z calcd. for C$_{30}$H$_{19}$O$_7$ ([M-H]$^-$): 491.1136, found 491.1149.

Fl-3OMe: flaxen powder, yield: 87.67 %.

$^1$H NMR (400 MHz, CDCl$_3$, TMS) δ 8.03 (d, $J = 6.8$ Hz, 1H), 8.03 (d, $J = 6.8$ Hz, 1H), 7.87 (d, $J = 16$ Hz, 1H), 7.74 (quint, $J = 7.2$ Hz, 2H), 7.35 (s, 1H), 7.19 (d, $J = 7.6$ Hz, 1H), 7.08-7.12 (m, 2H), 6.99 (d, $J = 7.6$ Hz, 1H), 6.80-6.87 (m, 2H), 6.69 (s, 1H), 6.64 (s, 1H), 6.61 (d, $J = 5.2$ Hz, 1H), 6.53 (d, $J = 8.8$ Hz, 1H), 6.16 (s, 1H), 3.86 (s, 3H).

$^{13}$C NMR (100 MHz, (CD$_3$)$_2$CO, TMS) δ 168.5, 164.4, 160.2, 159.6, 152.9, 152.6, 152.4, 152.2, 151.8, 146.9, 135.5, 135.4, 130.1, 130.0, 129.3, 129.1, 126.6, 124.7, 124.1, 121.2, 117.9, 117.1, 117.1, 113.0, 110.4, 110.3, 102.6, 82.0, 54.8. EMS/MS m/z calcd. for C$_{30}$H$_{19}$O$_7$ ([M-H]$^-$): 491.1136, found 491.1149.

Fl-4OMe: yellow powder, yield: 85.42 %.

$^1$H NMR (400 MHz, CDCl$_3$, TMS) δ 8.02 (d, $J = 6.0$ Hz, 1H), 7.86 (d, $J = 6.8$ Hz, 1H), 7.63 (quint, $J = 5.8$ Hz, 2H), 7.56 (d, $J = 8.8$ Hz, 2H), 7.15 (s, 1H), 7.04 (d, $J = 5.6$ Hz, 1H), 6.95 (d, $J = 8.8$ Hz, 2H), 6.79-6.86 (m, 2H), 6.67 (s, 1H), 6.62 (d, $J = 8.4$ Hz, 1H), 6.48-6.54 (m, 2H), 6.31 (s, 1H), 3.86 (s, 3H). $^{13}$C NMR (100 MHz, (CD$_3$)$_2$CO, TMS) δ 168.51, 164.67, 162.11, 159.57, 152.92, 152.51, 152.20, 151.72, 146.74, 135.39, 130.39, 130.09, 129.82, 129.31, 129.00, 126.75, 126.63, 124.67, 124.09, 117.93, 116.96, 114.45, 114.30, 113.93, 112.82, 110.31, 102.53, 81.99, 54.95. EMS/MS m/z calcd. for C$_{30}$H$_{19}$O$_7$ ([M-H]$^-$): 491.1136, found 491.1173.

Fl-2NO$_2$: yellow powder, yield: 74.43 %.

$^1$H NMR (400 MHz, (CD$_3$)$_2$CO, TMS) δ 9.11 (s, $J = 6.0$ Hz, 1H), 8.28 (d, $J = 16$ Hz, 1H), 8.15 (d, $J = 8.0$ Hz, 1H), 8.04 (t, $J = 8.0$ Hz, 2H), 7.86 (q, $J = 7.6$ Hz, 2H), 7.77 (t, $J = 8.4$ Hz, 2H), 7.38 (d, $J = 8.4$ Hz, 1H), 7.30 (s, 1H), 7.03 (d, $J = 6.4$ Hz, 1H), 6.95
(d, J = 8.4 Hz, 1H), 6.81 (t, J = 8.0 Hz, 2H), 6.70 (q, J = 8.8 Hz, 2H). 13C NMR (100 MHz, (CD3)2CO, TMS) δ 168.45, 163.68, 159.60, 159.49, 152.91, 152.17, 151.76, 148.81, 142.08, 135.40, 133.86, 131.21, 130.11, 129.56, 129.43, 129.32, 129.16, 126.62, 124.84, 124.67, 124.10, 121.48, 117.77, 117.35, 112.86, 112.78, 110.35, 110.26, 102.52, 102.46, 81.88. EMS/MS m/z calcd. for C29H16NO8 ([M-H]): 506.0881, found 506.0913.

Fl-3NO2: yellow powder, yield: 72.94%.

1H NMR (400 MHz, (CD3)2CO, TMS) δ 9.81 (s, 1H), 8.24 (s, 1H), 7.87 (t, J = 8.0 Hz, 2H), 7.62 (d, J = 10.0 Hz, 1H), 7.59 (s, 1H), 7.38 (t, J = 7.2 Hz, 1H), 7.31 (t, J = 7.6 Hz, 2H), 6.92 (t, J = 10.0 Hz, 2H), 6.62 (d, J = 16.0 Hz, 1H), 6.57 (d, J = 8.8Hz, 1H), 6.42 (d, J = 8.4, 1H), 6.29 (s, 1H), 6.17 (s, 2H). 13C NMR (100 MHz, (CD3)2CO, TMS) δ 168.5, 164.0, 159.7, 152.9, 152.2, 152.2, 151.8, 148.9, 144.3, 143.5, 136.0, 135.4, 134.2, 130.4, 129.4, 129.3, 129.2, 126.6, 124.9, 124.7, 124.5, 124.4, 124.1, 123.8, 123.0, 120.8, 120.0, 118.3, 117.8, 117.3, 112.9, 110.3, 102.6. EMS/MS m/z calcd. for C29H16NO8 ([M-H]): 506.0881, found 506.0931.

Fl-4NO2: yellow powder, yield: 76.57 %.

1H NMR (400 MHz, (CD3)2CO, TMS) 10.28 (s, 1H), 8.31 (d, J = 8.8 Hz, 2H), 8.14 (d, J = 8.8 Hz, 1H), 8.06 (t, J = 7.2 Hz, 2H), 7.87-7.76 (m, 3H), 7.39 (s, 2H), 7.17 (d, J = 16 Hz, 1H), 7.04 (d, J = 6.8 Hz, 1H), 6.89 (d, J = 8.8 Hz, 1H), 6.76 (s, 1H), 6.43 (s, 2H). 13C NMR (100 MHz, (CD3)2CO, TMS) δ 168.50, 163.83, 159.59, 152.89, 152.16, 151.74, 148.82, 144.10, 144.03, 140.31, 135.41, 130.12, 129.49, 129.30, 129.14, 126.60, 124.70, 124.07, 124.01, 121.13, 117.76, 117.30, 112.87, 110.37, 110.25, 102.54, 81.92. EMS/MS m/z calcd. for C29H16NO8 ([M-H]): 506.0881, found 506.0933.

1.3 Theoretical simulation method

DFT calculations have been applied to simulate the recognition process to compare the properties of these probes. All the calculations were performed with functional B3LYP36 for stable structures and UB3LYP for free radicals, with a combination of double-ζ quality consisting of 6-31G* for C, H elements and 6-31+G** for N, O, S elements on Gaussian 09 Program37. The optimized structures were confirmed to be local minimums due to the non-existence of imaginary frequency. The environmental effect was included via the PCM model with water as the solvent molecule.

1.4 Solution preparation procedure

All the reagent grade chemicals consumed in this work were procured from commercial sources and used as received. Stock solutions of all probes (200 μM) were
prepared in DMSO. Stock solutions of all analytes (2 mM) were prepared in 0.1 M PBS, pH=7.4.

To a 10 mL volumetric tube, 1 mL of 200 µM probe and different volume of GSH (2 mM in 0.1M PBS) were added. The mixture was diluted to 10 mL with 0.1 M PBS. Then, 3.0 mL of each solution was transferred to a 1 cm quartz cell. The fluorescence data was recorded between 480 nm-700 nm. The excitation and emission wavelength of slit width were both set at 5.0 nm and the excitation wavelength was set at 470 nm.

2. Optical properties of designed GSH probes

2.1 pH stability

![Figure S1. pH stability of Fl-H](image)

![Figure S2. pH stability of Fl-2OMe](image)
Figure S3. pH stability of Fl-3OMe

Figure S4. pH stability of Fl-4OMe
2.2 selectivity and competition experiments

Figure S5. selectivity and competition experiment of Fl-2OMe

Figure S6. selectivity and competition experiment of Fl-3OMe

Figure S7. selectivity and competition experiment of Fl-4OMe
2.3 Titration experiments

**Figure S8.** Fluorescence spectrum changes of Fl-2OMe upon addition of GSH (0-80 equiv.)

**Figure S9.** Fluorescence spectrum changes of Fl-3OMe upon addition of GSH (0-80 equiv.)

**Figure S10.** Fluorescence spectrum changes of Fl-4OMe upon addition of GSH (0-80 equiv.)
2.4 linear relationship between probes and GSH

Figure S11. Linear relationship between Fl-2OMe and GSH (0-50 equiv.)

Figure S12. Linear relationship between Fl-3OMe and GSH (0-50 equiv.)

Figure S13. Linear relationship between Fl-4OMe and GSH (0-50 equiv.)
2.5 Time-depended experiments

**Figure S14** Time-depended experiment of Fl-H in room temperature, 37 °C and 37 °C with esterase.

**Figure S15** Time-depended experiment of Fl-2OMe in room temperature, 37 °C and 37 °C with esterase.

**Figure S16** Time-depended experiment of Fl-3OMe in room temperature, 37 °C and 37 °C with esterase.

**Figure S17** Time-depended experiment of Fl-4OMe in room temperature, 37 °C and 37 °C with esterase.
3. Photo stability designed GSH probes

3.1 Photo stability in-vitro

**Figure S18** Photo stability of Fl-H in-vitro. a) Fluorescent intensity changes; b) LC changes. Under illumination for 10 days with a total illuminance of $1.2 \times 10^6$ lux·hr.

**Figure S19** Photo stability of Fl-2OMe in-vitro. a) Fluorescent intensity changes; b) LC changes. Under illumination for 10 days with a total illuminance of $1.2 \times 10^6$ lux·hr.

**Figure S20** Photo stability of Fl-3OMe in-vitro. a) Fluorescent intensity changes; b) LC changes. Under illumination for 10 days with a total illuminance of $1.2 \times 10^6$ lux·hr.

**Figure S21** Photo stability of Fl-4OMe in-vitro. a) Fluorescent intensity changes; b) LC changes. Under illumination for 10 days with a total illuminance of $1.2 \times 10^6$ lux·hr.
3.2 Photo stability in cells

**Figure S22** Photo stability scatter diagram of Fl-H under illumination in cells by flow cytometry.

**Figure S23** Photo stability scatter diagram of Fl-2OMe under illumination in cells by flow cytometry.
Figure S24 Photo stability scatter diagram of Fl-3OMe under illumination in cells by flow cytometry.

Figure S25 Photo stability scatter diagram of Fl-4OMe under illumination in cells by flow cytometry.
4. Cytotoxicity test

Figure S26. Cell viability on different concentration.

5. Living mice imaging

The positive cell imaging results in MCF-7 cells encouraged us to pursue the suitability of our probes for monitoring GSH in living animals. We used Kunming mice as a model to assess the effectiveness of using probe Fl-H/2/3/4OMe to detect GSH in a living organism (Figure S15-S18). Taking Fl-H as an example, the mouse was given a hypodermic injection with the pure probe (0.5 mM, in 200 μL PBS buffer solution). After 5 min., an obvious enhancement of fluorescence was observed. The signal intensity increased gradually with time and tended to be stable after about 60 min. By comparison, mice treated with NEM (2 mM, in 100 μL PBS buffer solution) before probe injection showed only a negligible fluorescent emission. Mice treated with α-LPA (2 mM, in 100 μL PBS buffer solution) prior to probe injection exhibited more intense fluorescence than the probe-only group. These results suggested that Fl-H/2/3/4OMe are sensitive enough to detect basal levels of endogenous GSH produced without stimulation, and could tolerate abnormal GSH concentrations. These experiments have highlighted the utility of Fl-H/2/3/4OMe for quantitative detection of GSH in living organisms.
**Figure S27.** Living Mice imaging of probe FL-H +50 equiv GSH in 60 min. a) Mice inject with 1 mM probe FL-H; b) Mice inject with 2 mM NEM, then inject with 1 mM probe FL-H; c) Mice inject with 2 mM α-LPA, then inject with 1 mM probe FL-H.

**Figure S28.** Living Mice imaging of probe FL-2OMe +50 equiv GSH in 60 min. a) Mice inject with 1 mM probe FL-2OMe; b) Mice inject with 2 mM NEM, then inject with 1 mM probe FL-2OMe; c) Mice inject with 2 mM α-LPA, then inject with 1 mM probe FL-2OMe.
**Figure S29.** Living Mice imaging of probe Fl-3OMe + 50 equiv GSH in 60 min. a) Mice injected with 1 mM probe Fl-3OMe; b) Mice inject with 2 mM NEM, then inject with 1 mM probe Fl-3OMe; c) Mice inject with 2 mM α-LPA, then inject with 1 mM probe Fl-3OMe.

**Figure S30.** Living Mice imaging of probe Fl-3OMe + 50 equiv GSH in 60 min. a) Mice injected with 1 mM probe Fl-4OMe; b) Mice inject with 2 mM NEM, then inject with 1 mM probe Fl-4OMe; c) Mice inject with 2 mM α-LPA, then inject with 1 mM probe Fl-4OMe.
6. Computational details

DFT calculations have been applied to simulate the recognition process by comparing the properties of these probes. All the calculations were performed with B3LYP functional\(^1\) and UB3LYP with a combination of basis of double-\(\zeta\) quality consisting of 6-31G** for C, H elements, 6-31+G** for N, O, S elements on Gaussian 09 Program\(^2\). The optimized structures were confirmed to be local minimums due to the non-existence of imaginary frequency, and the environmental effect was included via PCM model with water as the solvent molecule.

![Figure S31. Calculated structure of designed GSH probes.](image)

All these subsequent calculation such as front orbital theory analysis, energies analysis, Fukui function and NBO analysis were all obtained based on these structures.
7. NMR, MS spectra of all synthesized compounds

7.1 NMR spectra of all probes

Figure S32. $^1$H NMR of Fl-H in d$_6$-DMSO

Figure S33. $^{13}$C NMR of Fl-H in d$_6$-DMSO
Figure S34. $^1$H NMR of Fl-α-Me in (CD$_3$)$_2$CO

Figure S35. $^{13}$C NMR of Fl-α-Me in (CD$_3$)$_2$CO
Figure S36. $^1$H NMR of Fl-$\beta$-Me in CDCl$_3$

Figure S37. $^{13}$C NMR of Fl-$\beta$-Me in CDCl$_3$
Figure S38. $^1$H NMR of Fl-2OMe in CDCl$_3$

Figure S39. $^{13}$C NMR of Fl-2OMe in (CD$_3$)$_2$CO
Figure S40. $^1$H NMR of Fl-3OMe in CDCl$_3$

Figure S41. $^{13}$C NMR of Fl-3OMe in (CD$_3$)$_2$CO
Figure S42. $^1$H NMR of Fl-4OMe in CDCl$_3$

Figure S43. $^{13}$C NMR of Fl-4OMe in (CD$_3$)$_2$CO
Figure S44. $^1$H NMR of Fl-2NO$_2$ in (CD$_3$)$_2$CO

Figure S45. $^{13}$C NMR of Fl-2NO$_2$ in (CD$_3$)$_2$CO
Figure S46. $^1$H NMR of Fl-3NO$_2$ in (CD$_3$)$_2$CO

Figure S47. $^{13}$C NMR of Fl-3NO$_2$ in (CD$_3$)$_2$CO
Figure S48. $^1$H NMR of Fl-4NO$_2$ in d$_6$-DMSO

Figure S49. $^{13}$C NMR of Fl-4NO$_2$ in d$_6$-DMSO
7.2 HRMS spectra of all probes

Figure S50. HRMS spectra of Fl-H

Figure S51. HRMS spectra of Fl-α-Me
Figure S52. HRMS spectra of Fl-β-Me

Figure S53. HRMS spectra of Fl-2OMe
Figure S54. HRMS spectra of Fl-3OMe

Figure S55. HRMS spectra of Fl-4OMe
Figure S56. HRMS spectra of Fl-2NO₂

Figure S57. HRMS spectra of Fl-3NO₂
Figure S58. HRMS spectra of Fl-4NO₂

References:
