Supporting Information

Visualization of the cysteine level during Golgi stress using a novel Golgi-targeting highly specific fluorescent probe

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1. Materials and instruments

Unless otherwise stated, chemical reagents were purchased from commercial vendor and were used as received. Absorption spectra were carried out using a UV-3101PC spectrophotometer. Fluorescence emission spectra were performed using a Horiba FluoroMax-4 spectrophotometer. The slit width was 5.0 nm for both excitation and emission. High resolution mass spectra (HRMS) were carried out using a LC-MS2010A instrument. Fluorescence imaging of Cys in live HeLa cells and zebrafish were carried out on an Olympus FV1000-IX81 confocal fluorescence microscope.

2. Determination of the detection limit

The detection limit was calculated based on the fluorescence titration. The detection limit (DL) was calculated as follows:

\[ DL = \frac{3\sigma}{k}, \]

\[ \sigma = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n-1}} \]

where \( \sigma \) is the standard deviation of the blank solution, \( \bar{x} \) is the mean of the blank measures, \( x_i \) is the value of blank measures, \( n \) is the number of tested blank measures (\( n = 20 \)), and \( k \) is the slope between the fluorescence intensities versus the concentrations of Cys.

3. Preparation of probe Gol-Cys

3.1. Synthesis of compound 1

The preparation of compound 1 was carried out according to the previous report \((\text{ACS Med. Chem. Lett., 2019, 10, 954–959})\).
4,4,4-Trifluoro-1-phenyl-1,3-butanedione (1.00 g, 4.63 mmol) and 
m-phenylenediamine (0.50 g, 4.63 mmol) were dissolved in 10 mL 1,2-dichloroethane, 
and refluxed for 12 h at 80 °C. Then, the solvent was removed by rotary evaporation, 
and yellowish green solid was obtained. The crude product was further purified by 
silica column to get the green solid.

![Chemical structure](image)

**Scheme S1.** Synthesis of compound 1

### 3.2. Synthesis of probe Gol-Cys

1,1'-Thiocarbonyldiimidazole (576 mg, 2 mmol) was dissolved in anhydrous 
\(N,N\)-dimethylformamide (DMF, 6 mL), and the mixture was added slowly to the 
anhydrous DMF solution (5 mL) of compound 1 (576 mg, 2 mmol, Scheme S1) and 
triethylamine (1.214 g, 12 mmol). After the mixture was stirred overnight at room 
temperature, 1-mercaptopropane (305 mg, 4 mmol) and DIPEA (1.35 g, 12 mmol) 
were added to the aforementioned solution. Then, the mixture was further stirred for 
24 h at room temperature. The mixture was extracted with \(\text{CH}_2\text{Cl}_2\) and dried with 
anhydrous \(\text{Na}_2\text{SO}_4\). The organic phase was removed under vacuum. The residue was 
purified by silica column with petroleum ether/\(\text{CH}_2\text{Cl}_2\) (1:1, v/v) as the eluent, and 
**Gol-Cys** was obtained as a white solid. \(^1\text{H NMR}\) (400 MHz, CDCl\(_3\)) \(\delta\) (ppm): 1.053(t, 
\(J = 7.2\) Hz, 3H), 1.738-1.829(m, 2H), 3.338(t, \(J = 7.6\) Hz, 2H), 7.502-7.579(m, 3H), 
7.851(d, \(J = 8.8\) Hz, 1H), 8.120-8.198(m, 4H), 8.486(d, \(J = 2.0\) Hz, 1H), 8.968(s, 1H);
13C NMR (100 MHz, CDCl3) δ (ppm): 13.53, 22.05, 38.39, 115.90, 115.96, 120.19, 123.47, 124.73, 127.60, 129.10, 130.38, 134.90, 135.22, 135.54, 137.99, 140.06, 149.53, 157.58. HRMS (ESI): Calcd for C20H18F3N2S2 [M+H]+ 407.0858; Found, 407.0858.

![Scheme S2. Synthesis of probe Gol-Cys](image)

4. General method

The fluorescence data were recorded in the aqueous solution (PBS, 10 mM, pH 7.4, 30% ethanol). Unless otherwise stated, the emission of probe Gol-Cys at 515 nm was measured after adding Cys in solution for 30 min at room temperature, and λex = 390 nm, slit widths: d_ex = d_em = 5 nm.

5. UV-vis absorption spectra
**Figure S1.** Absorption responses of probe **Gol-Cys** (5 μM) in the presence of Cys (20 μM) at 25 °C.

6. **Time-dependent fluorescence spectra of probe Gol-Cys for Cys**

![Time-dependent fluorescence spectra of probe Gol-Cys for Cys](image1)

**Figure S2.** Time-course of probe **Gol-Cys** (5 μM) for tracing Cys (20 μM) at 25 °C.

7. **Fluorescence titration of probe Gol-Cys**

![Fluorescence titration of probe Gol-Cys](image2)
**Figure S3.** The fluorescence intensities at 515 nm of probe **Gol-Cys** (5 μM) with the increasing concentrations of Cys (0-20 μM) at 25 °C.

8. The selectivity of probe Gol-Cys

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**Figure S4.** The fluorescence responses of probe **Gol-Cys** (5 μM) in the presence of various analytes (100 μM except for specific labels). 1. Blank, 2. H$_2$O$_2$, 3. TBHP, 4. 'O'Bu, 5. O$_2^-$, 6. $^1$O$_2$, 7. OH', 8. ONOO', 9. NO, 10. HOCl, 11. Cys (20 μM). Bars represent the fluorescence intensities at 515 nm and each spectrum was acquired 30 min after various analytes addition at 25 °C.
Figure S5. The fluorescence responses of probe \textbf{Gol-Cys} (5 μM) for monensin (Mone, 100 μM) and Cys (20 μM).


Figure S6. Photo-stability of \textbf{Gol-Cys} (black) and compound 1 (red) from 0 to 30 min of time-sequential scanning.
Figure S7. Photo-stability of Gol-Cys (red) and compound 2 (blue) from 0 to 30 min of time-sequential scanning.

10. Live subject statement

All experimental procedures were carried out in strict accordance with the National Institute of Health (NIH) guidelines for the Care and Use of Laboratory Animals and the regulations of Qilu University of Technology on the ethical use of animals. All experimental procedures were approved by the faculty Ethical Committee of the Biology Institute of the Shandong Academy of Sciences. All efforts were made to minimize the number of animals used and their suffering. The sources of biological samples in our experiments were all from Shanghai Institute of Biochemistry and Cell Biology, China Academy of Sciences (Shanghai, China).

11. Cytotoxicity assays

The cell viability of HeLa cells, treated with probe Gol-Cys, was assessed by a cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Tokyo, Japan). Briefly, HeLa cells, seeded at a density of $1 \times 10^6$ 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 HeLa cells were incubated with various concentrations (0, 5, 10, 20 and 30 μM) of probe Gol-Cys suspended in culture medium for 12 h. Subsequently, CCK-8 solution was added into each well for 2 h, and absorbance at 390 nm was measured.
Figure S8. HeLa cells viability with different concentration of probe Gol-Cys.

12. Imaging studies of live cells

The HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and incubated under an atmosphere containing 5% CO₂ at 37 °C humidified air for 24 h. DMEM contains 10% fetal bovine serum and 1% penicillin-streptomycin.

Control cells were imaged by confocal fluorescence microscope. Then, probe Gol-Cys (10 μM) was used to incubate HeLa cells for 40 min, the culture medium was then removed and rinsed with phosphate buffer saline for three times before fluorescence imaging was performed. The other groups of cells were incubated with Cys (100 μM) for 30 min, washed with culture water, and then treated with probe Gol-Cys (10 μM) for 40 min for imaging. Then, in order to confirm that probe Gol-Cys can detect basal Cys, another cells were incubated with NEM (200 μM) for 30 min, washed with culture water, and then treated with probe Gol-Cys (10 μM) for 40 min. Then the fluorescence imaging of cells was carried out by confocal fluorescence microscope.
13. 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 CaCl₂, 0.16 mM MgSO₄) and cultured in illumination incubator at 28 °C.

To ascertain whether probe Gol-Cys can produce a marked effect in imaging Cys in vivo, we used probe Gol-Cys to visualize Cys in five-day-old zebrafish. Besides the imaging of control zebrafish, the 5-day-old zebrafish were incubated with probe Gol-Cys (10 µM) for 40 min, the culture water was used to clean and remove the residual probe, and then confocal fluorescence microscopy was used to observe. In addition, one group of zebrafish was treated with Cys (100 µM) for 30 min, washed with culture water, and then incubated with probe Gol-Cys (10 µM) for 40 min before imaging with confocal microscope. Another group was treated with NEM (200 µM) for 30 minutes, washed with culture medium, and then incubated with probe Gol-Cys (10 µM) for 40 min. Finally, the fluorescence imaging of zebrafish was carried out by confocal fluorescence microscope.

As designed in the cell experiment, the probe-treated zebrafish exhibited obvious fluorescence enhancement than zebrafish in the control group, attributing to that the probe could detect native Cys in zebrafish (Figure S9a-b). Next, the zebrafish pretreated with Cys make a significant contrast in fluorescence with the probe loaded
zebrafish, indicating that the presence of Cys in zebrafish could induce the enhancement of fluorescence signal (Figure S9c). Finally, after the removal of Cys by NEM pretreatment, the fluorescence intensity of zebrafish was reduced obviously. This may be attributed to the reduction of Cys levels (Figure S9d). Therefore, these images demonstrated the utility of probe **Gol-Cys** in imaging native Cys levels in intricate biological system.

**Figure S9.** Fluorescence images of zebrafish. (a) Control zebrafish. (b) Zebrafish treated with **Gol-Cys** (10 μM) for 40 min. (c) Zebrafish pretreated with Cys (100 μM) for 30 min, and then treated with **Gol-Cys** (10 μM) for 40 min. (d) Zebrafish pretreated with NEM (200 μM) for 30 min, and then treated with **Gol-Cys** (10 μM) for 40 min. (e) Fluorescence intensities of relative fluorescence intensities from the corresponding zebrafish.

14. **Preparation of reactive oxygen species (ROS) and reactive nitrogen species (RNS)**

**TBHP**
*tert*-butylhydroperoxide (TBHP) were diluted from the commercially available solution to 0.1 M in ultrapure water.

\[^\cdot OH\]

Hydroxyl radical (\(^\cdot OH\)) was generated by Fenton reactions. To prepare \(^\cdot OH\) solution, hydrogen peroxide (\(\text{H}_2\text{O}_2\), 2 eq) was added to \(\text{FeSO}_4\) in the deionised water.

\[^\cdot O\text{Bu}\]

*tert*-butoxy radical (\(^\cdot\text{O}\text{Bu}\)) was generated by Fenton reactions.

\[^{1}\text{O}_2\]

Superoxide (\(^{1}\text{O}_2\)) was generated from \(\text{K}_2\text{O}\) in DMSO.

\[^{1}\text{O}_2\]

Singlet oxygen (\(^{1}\text{O}_2\)) was generated from HOCl and \(\text{H}_2\text{O}_2\).

\(\text{NO}\)

Nitric oxide (NO) was generated from potassium nitroprusside dihydrate.

\(\text{H}_2\text{O}_2\)

The concentration of hydrogen peroxide (\(\text{H}_2\text{O}_2\)) was determined from the absorption at 240 nm \((\varepsilon = 43.6 \text{ M}^{-1} \text{ cm}^{-1})\).

\(\text{NaOCl}\)

The concentration of sodium hypochlorite (NaOCl) was determined from the absorbance at 292 nm \((\varepsilon = 350 \text{ M}^{-1} \text{ cm}^{-1})\).

\(^{\cdot\text{ONOO}^-}\)

Simultaneously, 0.6 M \(\text{KNO}_2\), 0.6 M HCl and 0.7 M \(\text{H}_2\text{O}_2\) was added to a 3 M NaOH solution at 0 °C. The concentration of peroxynitrite was estimated by using the
extinction co-efficient of 1670 cm\(^{-1}\) M\(^{-1}\) at 302 nm in 0.1 M sodium hydroxide aqueous solutions.

15. Imaging studies of localization experiments

In the Golgi localization experiment, chemical reagents for targeting Golgi, mitochondria, lysosomes and endoplasmic reticulum were obtained from commercial suppliers. BODIPY TR Ceramide was used as a commercial Golgi targeting dye (\(\lambda_{\text{ex}} = 594\) nm; \(\lambda_{\text{em}} = 600-700\) nm). MitoTracker Red CMXRos was used as mitochondrial dye (\(\lambda_{\text{ex}} = 578\) nm; \(\lambda_{\text{em}} = 590-630\) nm). LysoTracker Red DND-99 was used as lysosomal dye (\(\lambda_{\text{ex}} = 559\) nm; \(\lambda_{\text{em}} = 580-620\) nm). ER-Tracker Red was used as endoplasmic reticulum dye (\(\lambda_{\text{ex}} = 594\) nm; \(\lambda_{\text{em}} = 600-660\) nm).

In the localization experiments, HeLa cells were divided into four groups. Each group of cells was pretreated with 100 \(\mu\)M Cys for 30 min. Then, each group of cells was treated with probe \textbf{Gol-Cys} (10 \(\mu\)M) and BODIPY TR Ceramide, MitoTracker Red CMXRos, LysoTracker Red DND-99 and ER-Tracker Red respectively for 40 min. Finally, the fluorescence imaging of cells was carried out by confocal fluorescence microscope.

16. Imaging studies of Golgi stress response

Before imaging by confocal fluorescence microscope, HeLa cells were divided into four groups. Each group was treated with different concentrations monensin (0, 0.5, 1, 2 \(\mu\)M) for 18 h. Then, each group cells was washed with culture water, and treated with probe \textbf{Gol-Cys} (10 \(\mu\)M) for 40 min. Finally, the fluorescence imaging of cells was carried out by confocal fluorescence microscope.
Before imaging by confocal fluorescence microscope, zebrafish were divided into four groups. Each group was treated with different concentrations monensin (0, 0.5, 1, 2 µM) for 18 h. Then, each group zebrafish was washed with culture water, and treated with probe Gol-Cys (10 µM) for 40 min. Finally, the fluorescence imaging of cells was carried out by confocal fluorescence microscope.

17. Characterization data of probe Gol-Cys

![Figure S10. ¹H-NMR data of probe Gol-Cys.](image-url)
18. Proofs for the reaction mechanism of probe Gol-Cys and Cys
Figure S13. $^1$H-NMR data of compound 1.

Figure S14. $^{13}$C-NMR data of compound 1.
Figure S15. HRMS data of the compound 1.

Figure S16. HRMS data of the compound 2.