NJC

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: G. Xu, Y. Tang and W. Lin, *New J. Chem.*, 2018, DOI: 10.1039/C8NJ01793J.

ROYAL SOCIETY OF CHEMISTRY

rsc.li/njc

View Article Online
DOI: 10.1039/C8NJ01793J

Journal Name

ARTICLE

A multi-signal fluorescent probe for discrimination of cysteine/homocysteine, and glutathione and the application in living cells and zebrafish

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Gaoping Xu, Yonghe Tang, and Weiying Lin*

Biological thiols are ubiquitous common in living organisms and play an important role in metabolism and redox homeostasis. Abnormal concentrations of biological thiols may lead to many malignant diseases. It is difficult to distinguish biological thiols by fluorescent techniques because they possess similar chemical structures and properties. Herein, we developed a new fluorescent probe **CI** to distinguish cysteine/homocysteine (Cys/Hcy) and glutathione (GSH) by multi-signal fluorescence mode. When the respective biological thiols were introduced, the probe would react with these biological thiols by thiol-halogen S_NAr nucleophilic substitution-rearrangement mechanism and display two different fluorescent signals with two different excitation wavelengths. After identifying Cys/Hcy and GSH, **CI** showed good two-photon property because of the coumarin structure. Significantly, the probe was effectively applied for the imaging of Cys/Hcy and GSH in living cells via two-color fluorescence imaging.

Introduction

Biological thiols, including glutathione (GSH), cysteine (Cys) and homocysteine (Hcy), play an important role in signal transduction and chelation of metal ions[1-4]. In organisms, GSH, as the most abundant intracellular biological thiol, is mainly replenished by three essential amino acids, Cys, glycine (Gly), and glutamate (Glu)[5-6]. Cys and Hcy, with the similar structure, are endogenously produced from methionine and serine. However, the change of biological thiols concentration is related to many diseases. Abnormal concentrations of GSH in cells may lead to cancer, neurodegenerative disorders, cystic fibrosis (CF), HIV[7]. The abnormality of Cys is related to many syndromes, including liver damage, lethargy, edema[8]. When Hcy concentration changes, it often leads to Alzheimer's disease, cardiovascular diseases (CVD), cognitive impairment, folate and cobalamin (vitamin B12) deficiency, birth defects[9-14]. These three biological thiols have similar structures, which is a major hindrance to research their physiological functions and related diseases caused by the biological thiols. Therefore, it is important to develop effective methods to identify Cys/Hcy and GSH in biological systems simultaneously.

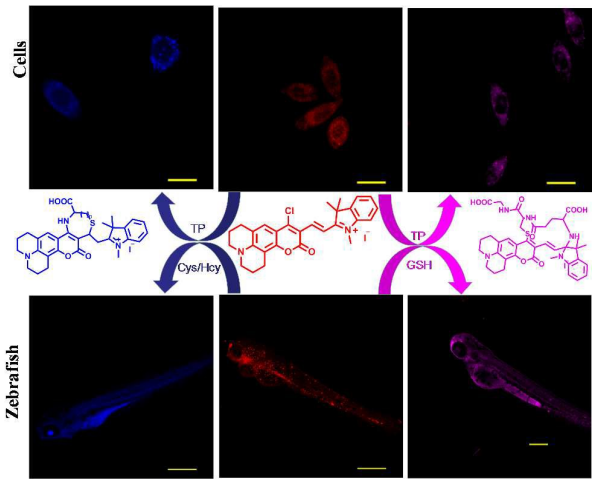
So far, many existing strategies for the detection of these biological thiols, such as colorimetric method, electrochemical method, high-performance liquid chromatography (HPLC), capillary electrophoresis, liquid chromatography, have been developed in the previous reports[15-19]. However, these strategies couldn't achieve the continuous monitoring of biological thiols and need sophisticated professional instruments and strictly required operating techniques, which require a lot of money and time. In comparison, Fluorescent probes have attracted considerable attention due to the high sensitivity and selectivity, less consumption, low cost, and good reproducibility[20-25]. Particularly, the two-photon (TP) fluorescence imaging methods show many advantages, such as higher spacial resolution, deep penetration, and low background signals[26-28]. Nowadays, there are many fluorescent probes sensing of biological thiols have been reported[29-38]. While, there are still many challenges for the probes of biological thiols. Most biological thiols fluorescent probes possessed the short emission wavelengths (less than 600 nm) in one-photon modes (OP), which limit their bio-imaging application due to the interference of spontaneous fluorescence of living cells. The emission shifts of some of these biological thiols probes were relatively small, which is disturbing the detection of biological thiols because the emission spectra have overlaps before and after the interaction between the fluorescent probe and biological thiols. Importantly, most these fluorescent probes were difficult to distinguish between these biological thiols because of the structural similarity of the biological thiols. Thus, it is crucial to develop new TP probes with a large emission shift for biological thiols detection in living systems.

Thereby, we developed a near infrared (NIR) fluorescent probe, named as **CI**, with the ability to convert NIR to TP modes after detecting Cys/Hcy and GSH. The structure of **CI** was a coumarin-indole π-conjugated system (semi-cyanine structure) with emission at 675 nm. When Cys/Hcy or GSH was introduced, the π-conjugated system of **CI** was destroyed basing on the thiol-halogen S_NAr

Institute of Fluorescent Probes for Biological Imaging, School of Chemistry and Chemical Engineering, School of Materials Science and Engineering, University of Jinan, Jinan, Shandong 250022, P. R. China. E-mail: weiyinglin2013@163.com
†Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

View Article Online
DOI: 10.1039/C8NJ01793J

nucleophilic substitution-rearrangement reaction. As a result, the blue emission (475 nm, **CI** reacted with Cys/Hcy) or the red emission (575 nm, **CI** reacted with GSH) of the classical TP coumarin dyes were detected respectively. Therefore, the unique design of the probe may satisfy the need for imaging Cys/Hcy and GSH in TP modes with multi-signal. The probe **CI** could distinguish Cys/Hcy and GSH with different fluorescent emission. Significantly, the innovative probe **CI** was applied for the detection of Cys/Hcy and GSH in living HeLa cells with the OP and TP modes and zebrafish with the OP modes.



Scheme 1. The proposed response mechanism of probe **CI** with respective biological thiols.

Experimental Section

Materials and instruments

All raw materials and reagents from supplier were directly used without further purification. Absorption spectra were tested by a UV-vis spectrophotometer (Shimadzu UV-2600, Japan). Fluorescent spectra were tested by a fluorescence spectrophotometer (HITACHI F4600, Japan). The fluorescence imaging of cells and zebrafish were tested using a confocal microscope (Nikon Eclipse Ti-e, Japan). The pH measurements were performanced on a pH meter (Mettler-Toledo Delta 320, Switzerland). High-resolution mass spectra (HRMS) were recorded on a mass spectrometer (Bruker Apex Ultra 7.0 T FTMS, Germany) in electrospray ionization (ESI) mode. NMR spectra were recorded on a 400 MHz Digital NMR Spectrometer (Bruker AVANCE III, Germany), using tetramethylsilane (TMS) as internal reference.

Preparation of the spectroscopic test

The solutions of various testing species were prepared with Hcy, GSH, NAC, Cys, Asp, Glu, His, Phe, Pro, Ser, Thr, Try, Tyr, NaCl, NaClO, $CuCl_2 \bullet 2H_2O$, $ZnSO_4$, H_2O_2, $HgCl_2$, $MgSO_4$, respectively. The concentration of the available stock solution (1.0 mM) of **CI** was prepared by dissolving the requisite amount of the probe in DMSO.

Cytotoxicity assay

The cytotoxicity of probe **CI** at different concentration (0, 5, 10, 20 and 30 μM) was evaluated by a standard MTT assay according to previous procedure[39].

Cell culture and bio-imaging biological thiols in living HeLa cells

The HeLa cells were cultured in 35 mm glass-bottom culture dishes with DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (Fetal Bovine Serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C for 24 h. Before the imaging experiments, the cells were washed with PBS for 2-3 times.

The imaging experiments were divided into five groups: the first group of cells were cultured with 500 μM N-ethylmaleimide (NEM, thiols scavenger reagent) for 30 min, subsequently incubated with 200 μM Cys for 15 min and 5 μM **CI** for 30 min; the second group of cells were cultured with 500 μM NEM for 30 min, then incubated with 200 μM Hcy for 15 min and 5 μM **CI** for 30 min; the third group of cells were cultured with 500 μM NEM for 30 min, then incubated with 200 μM GSH for 15 min and 5 μM **CI** for 30 min; the fourth group of cells were cultured with 500 μM NEM for 30 min, subsequently incubated with 5 μM **CI** for 30 min; the fifth group of cells were cultured with 5 μM **CI** for 30 min.

The one-photon parameter: DAPI Channel (Excitation wavelength: 405 nm; Emission band: 425-475 nm); TRITC Channel (Excitation wavelength: 488 nm; Emission band: 570-620 nm); Cy5 Channel (Excitation wavelength: 647 nm; Emission band: 663-738 nm).

The two-photon parameter: Excitation wavelength: 760 nm; Emission band: 425-475 nm, 570-620 nm.

Bio-imaging biological thiols in living zebrafish

Wild type zebra fishes were purchased from the *Nanjing EzeRinka Biotechnology Co., Ltd*. All procedures for this study were approved by the Animal Ethical Experimentation Committee of Shandong University according to the requirements of the National Act on the use of experimental animals (China). The imaging experiments were divided into five groups: The first three groups of zebrafish were cultured with 200 μM NEM for 30 min, subsequently incubated with 200 μM Cys, 200 μM Hcy and 200 μM GSH for 15 min respectively, and incubated with 5 μM **CI** for 30 min; the fourth group of zebrafish were cultured with 500 μM NEM for 30 min, subsequently incubated with 5 μM **CI** for 30 min; the fifth group of zebrafish were cultured with 5 μM **CI** for 30 min.

The one-photon parameter: DAPI Channel (Excitation wavelength: 405 nm; Emission band: 425-475 nm); TRITC Channel (Excitation wavelength: 488 nm; Emission band: 570-620 nm); Cy5 Channel (Excitation wavelength: 647 nm; Emission band: 663-738 nm).

Synthetic procedures and methods

Synthesis of compound CI. The synthetic routes of compound **1**, compound **2**, compound **3** and compound **4** were shown in supporting information. Compound **3** (1.9 g, 10.0 mmol), compound **4** (2.6 mL, 12.0 mmol) and piperidine (1 mL, 10.0 mmol) were added to a solution of 20 mL of ethanol and refluxed for 8 h. Then the solvent was removed and the product was purified by column chromatography with dichloromethane /methanol (20:1) to obtain the compound **CI** of 1.8 g. ^{1}HNMR (400 MHz, DMSO-d_6) :δ 8.37 (d, J = 15.4 Hz, 1H), 8.02 (d, J = 15.4 Hz, 1H), 7.87 (t, J = 7.2 Hz, 2H), 7.60 (p, J = 7.4 Hz, 2H), 7.50 (s, 1H), 3.96 (s, 3H), 3.56-3.44 (m, 4H), 2.80

View Article Online
DOI: 10.1039/C8NJ01793J

(td, J_1 = 18.8 Hz, J_2 =6.0 Hz, 4H), 1.93 (q, J = 6.2 Hz, 4H), 1.77 (s, 6H).[13]CNMR (100 MHz, DMSO-d_6): δ 181.14, 158.08, 152.64, 151.03, 150.65, 145.19, 143.29, 142.46, 129.47, 129.07, 125.05, 22.62, 115.03, 111.69, 108.20, 105.55, 51.86, 50.81, 50.20, 34.21, 27.18, 26.97, 20.71, 19.94, 19.67. HR-MS calcd for $[C_{28}H_{30}ClN_2O_2]$: 459.1841, found m/z 459.1849.

Results and Discussion

Design and synthesis of the fluorescent probe CI

The combination of two classical fluorescent dyes is one of the common strategies for developing new fluorescent dyes[40]. We directly connected the indole quaternary ammonium salt with the coumarin group, and introduced chlorine atom into the 4-position of coumarin, constructed the new compound **CI**. With the introduction of indole quaternary ammonium salts, coumarin group and indole quaternary ammonium salts constituted a strong conjugate system and the emission wavelength of compound **CI** was in the near infrared region. Previous studies have reported, indole quaternary ammonium salt, double bond, and chlorine atom were easy to react with nucleophilic reagents[41-47]. Biological thiols may react with different reaction locus because of the difference in structure and molecular size, releasing different fluorescent signals. When treated with Cys/Hcy and GSH, via thiol-halogen S_NAr nucleophilic substitution-rearrangement cascade reaction and nucleophilic addition reaction, the conjugation system of the probe was broken, then coumarin group, the classic two-photon dye, was released (Scheme 1). The probe **CI** was fully characterized by ^{1}H NMR (Fig. S1), ^{13}CNMR (Fig. S2) and HR-MS (Fig. S3).

Spectroscopic and optical property of the probe CI to Cys/Hcy and GSH

To evaluate the response ability of **CI** to Cys/Hcy and GSH, the absorption and fluorescence titration experiments was conducted in PBS (pH 7.4, contain 1% DMSO as co-solvent) at room temperature. As shown in the Fig. 1A, the free probe **CI** (5 μM) showed the maximum absorption at 614 nm (ε=41,200 M^{-1} cm^{-1}). After **CI** reacted with Cys/Hcy, the absorption peak of 614 nm disappeared and a new absorption peak appeared at about 380 nm. After **CI** reacted with GSH, the absorption peak of 614 nm disappeared and a new absorption peak appeared at about 480 nm. These phenomena indicated that Cys/Hcy and GSH may react with **CI** and break the original conjugate system of **CI**. The probe **CI** exhibited strong fluorescence emission at 675 nm (Fig. S4). Upon addition of Cys with different concentrations (0-40 μM) to **CI** solution, the fluorescence emission at 475 nm was gradually enhanced by stimulated with a light source of 380 nm (Fig. 1B), and the maximum fluorescence enhancement up to 8-fold (inset of Fig. 1B). The fluorescence titration experiment of Hcy also showed the same situation, and the fluorescence enhancement multiple was about six times (Fig. 1C and inset of Fig. 1C). When different concentrations GSH (0-40 μM) were introduced, the fluorescence emission at 575 nm was gradually enhanced, up to 15-fold (inset of Fig. 1D), by stimulated with a light source of 480 nm (Fig. 1D). The detection limit for Cys, Hcy and GSH were 0.014 μM, 0.081 μM, 0.097 μM, respectively basing on the fluorescence titration data. It is noted that the feature of the multi-signal and the low detection limit of **CI** may make it possible for the detection of Cys, Hcy and GSH in the living samples. We used HR-MS (Fig. S5-S7), time-dependent absorption spectra of the probe **CI** with NAC and DL-2-aminobutyricacid (Fig. S8) and ^{1}HNMR spectrum (Fig. S9) to verify the identification mechanism of **CI** for Cys, Hcy and GSH and obtained the detailed mechanism of the reaction of the probe with Cys/Hcy and GSH. For the detection of Cys/Hcy and GSH, the chlorine of **CI** was firstly replaced by sulfydryl from Cys/Hcy and GSH. Subquently, the amino group in the Cys/Hcy replaced the sulfydryl to form amino-substituted **CI**[48]. However, it was difficult for GSH to undergo the aforementioned intramolecular rearrangement to produce the corresponding amino-coumarinhemicyanine due to the unstable 10-membered macrocyclic transition state. Alternatively, the free amino group would attack sites 3 to produce the 14-membered ring products thio-coumarin (Fig S10). The results of HR-MS were consistent with the recognition mechanism proposed in scheme 1.

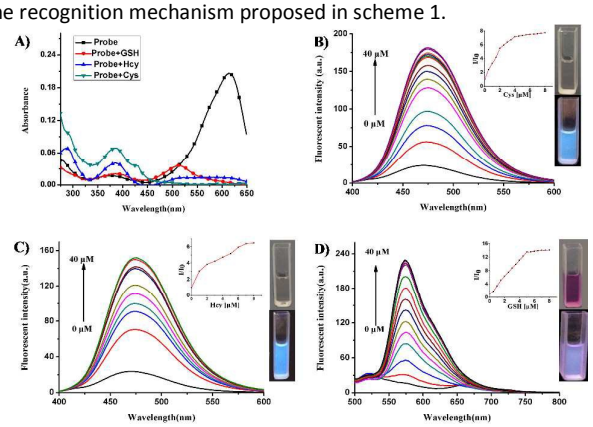


Fig. 1 The absorbance spectra and emission spectra of **CI** (5 μM) in the absence or presence of various biological thiols in PBS (pH 7.4, contain 1% DMSO as co-solvent). A) The absorbance spectra of **CI** (5 μM) in the absence or presence of various biological thiols; B) The emission spectra of **CI** (5 μM) in the presence of Cys (0-40 μM). Insets: the relationship between fluorescence intensity ratio (I/I_0) and the concentration of Cys; the color of **CI** in the presence Cys under the naked eye and the color of **CI** in the presence Cys under a 365 nm UV irradiation; C) The emission spectra of **CI** (5 μM) in the presence of Hcy (0-40 μM). Insets: the relationship between fluorescence intensity ratio (I/I_0) and the concentration of Hcy; the color of **CI** in the presence Hcy under the naked eye and the color of **CI** in the presence Hcy under a 365 nm UV irradiation; D) The emission spectra of **CI** (5 μM) in the presence of GSH (0-40 μM). Insets: the relationship between fluorescence intensity ratio (I/I_0) and the concentration of GSH; the color of **CI** in the presence GSH under the naked eye and the color of **CI** in the presence GSH under a 365 nm UV irradiation.

pH effect and kinetic studies

The pH effect tests about **CI** in the absence or presence of Cys, Hcy and GSH were carried out. As shown in Fig. S11-S13, **CI** was stable at wide range of pH value from 4.0 to 10.0 by stimulated with light sources of 614 nm. With the introduction of Cys, Hcy and GSH, the strong fluorescent signal enhancement changes were observed, and

View Article Online
DOI: 10.1039/C8NJ01793J

the fluorescence intensities were almost constant. These results illustrated **CI** is applicable to detection of Cys, Hcy and GSH in physiological pH environment. The response rate experiments of **CI** with Cys, Hcy and GSH were investigated (Fig. 2). Upon addition of Cys, the emission intensity reached the equilibrium state at about 25 min. Upon addition of Hcy, the emission intensity reached the equilibrium state at about 55 min. Upon addition of GSH, the emission intensity reached the equilibrium state at about 50 min. The results of kinetic experiments showed that **CI** has potential real-time imaging application value in living systems.

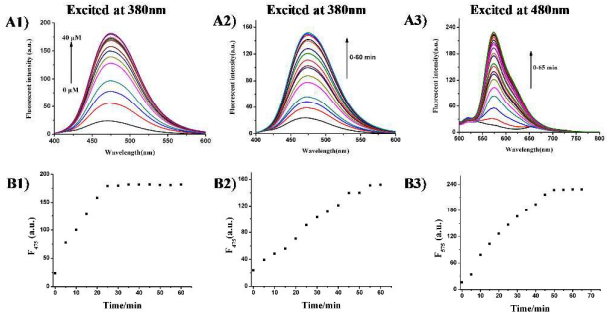


Fig. 2 The response rate experiments of **CI** (5 μM) in the presence of 40 μM Cys (A1, B1), 40 μM Hcy (A2, B2) and 40 μM GSH (A3, B3) in PBS (pH 7.4, contain 1% DMSO as co-solvent).

Selectivity and photo-stability studies

Probe was used to detect multiple substances (amino acids, metal ions, anions and reactive oxygen species) in study of the selectivity. As shown in Fig. 3, there was almost no effect on the fluorescence of **CI** treated with various interfering species. However, encounter of Cys/Hcy and GSH generated a significant change of fluorescence in the different channels. At the same time, we also have tested the photophysical responses of the probe with the coexist of Cys and GSH in the same concentration. The test results (Fig. S14) showed that the probe firstly reacts with Cys but not GSH with the coexist of Cys and GSH. The excellent character of **CI** indicated that the probe has the potential in bio-imaging. Furthermore, we also investigated the photo-stability of the probe **CI**. After **CI** was irradiated or non-irradiated by UV light whose wavelength is 365 nm, there was almost no change in the fluorescence intensity of the probe (Fig. S15-S17), indicating that **CI** would not be affected by the UV light and it may have potential application value in biological imaging.

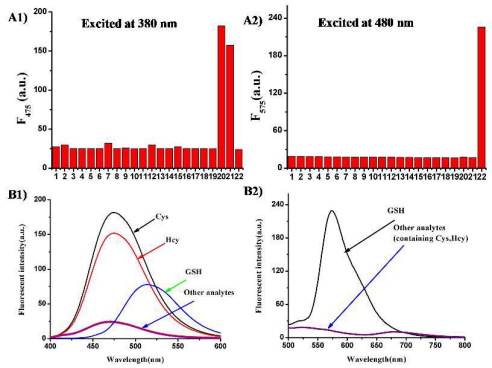


Fig. 3 The fluorescence intensity of **CI** (5 μM) in the presence of multiple interfering species in PBS (pH 7.4, contain 1% DMSO as co-solvent). 1: Probe; 2: Asp; 3: Glu; 4: His; 5: Phe; 6: Pro; 7: Ser; 8: Thr; 9: Try; 10: Tyr; 11: NaCl; 12: NaClO; 13: $CuCl_2 \bullet 2H_2O$; 14: $ZnSO_4$; 15: H_2O_2; 16: $HgCl_2$; 17: $MgSO_4$; 18: Na_3PO_4; 19: CH_3COOOH; 20: Cys; 21: Hcy; 22: GSH. 500 μM for others interfering species, 40 μM for Cys, 40 μM for Hcy, 40 μM for GSH. The fluorescence intensity at 475 nm, 575 nm were acquired with excitation at 380 nm, 480 nm respectively.

Fluorescence imaging for to Cys/Hcy and GSH in living cells

We tested the cytotoxicity of the probe **CI** for HeLa cells. As shown in the Fig. S18, the probe **CI** has low cytotoxicity for the HeLa cells, indicating that the probe could be used to imaging in living systems.

We then explored the ability of **CI** for imaging of Cys/Hcy and GSH in HeLa cells. Since there is a large amount of biological thiols in the organism, especially GSH, we used NEM to inhibit the biological thiols in cells before imaging. The cells were incubated with NEM (500 μM, 30 min), then incubated with Cys/Hcy (200 μM, 30 min) and 5 μM **CI** for 30 min in turn, significant fluorescent signals were observed in the DAPI channel by OP and TP modes (Fig. 4 a1, Fig. 4 b1, Fig. S19 a2, Fig. S19 b2). The cells exhibited significant fluorescent signals in the TRITC channel by OP and TP modes for GSH detection (Fig. 4 c2, Fig. S19 c3). The cells were only treated with **CI** showed a bright emission in TRITC channel by OP and TP modes due to the probe **CI** interacted with a high concentration of GSH in the cell (Fig. 4 d2, Fig. S19 d3). While, the cells pre-incubated with 500 μM NEM for 30 min, subsequently treated with 5 μM **CI** for 30 min showed strong fluorescence in Cy5 channel by OP modes (Fig. S19 e4) because NEM inhibited the activity of intracellular biological thiols. The cells imaging experiments showed that the probe **CI** could distinguish cellular Cys/Hcy and GSH using two different fluorescence signals in the cells imaging experiments.

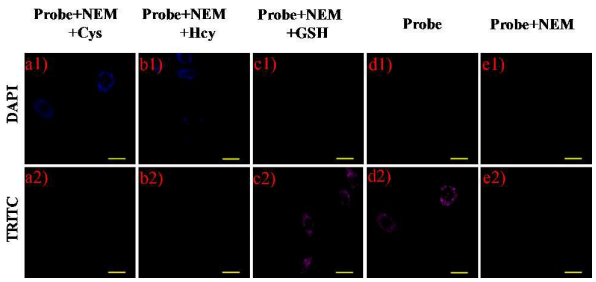


Fig. 4 Two-photon fluorescence imaging of probe **CI** (5 μM) responding to respective biological thiols in cells. a-c) The cells were incubated with NEM (500 μM) for 30 min, followed by addition of Cys (200 μM), Hcy (200 μM) and GSH (200 μM) respectively for 15 min, then incubated with **CI** (5 μM) for 30 min and imaged; d) The cells were incubated with **CI** (5 μM) for 30 min, then imaged; e) The cells were incubated with NEM (500 μM) for 30 min, followed by addition of **CI** (5 μM) for 30 min and imaged. Scale bar = 20 μm.

Fluorescence imaging for to Cys/Hcy and GSH in living zebrafish

Furthermore, we used zebrafish to image biological thiols. The zebrafish treated with NEM (500 μM, 30 min), Cys/Hcy (200 μM, 30

View Article Online
DOI: 10.1039/C8NJ01793J

min) and the probe **CI** (5 μM, 30 min) in turn exhibited different blue fluorescence (from DAPI channel) for Cys/Hcy (Fig. 5 a1, Fig. 5 b1) and red fluorescence (from TRITC channel) for GSH (Fig. 5c2). The zebrafish pre-treated with NEM (500 μM, 30 min), and incubated with **CI** (5 μM, 30 min) displayed the strong emission in Cy5 channel (Fig. 5 d3). Thus the zebrafish treated with **CI** (5 μM) exhibited strong fluorescence in TRITC channel and weak fluorescence in Cy5 channel (Fig. 5 e2, e3). The results indicated that probe **CI** also has the capacity to image Cys/Hcy and GSH in zebrafish.

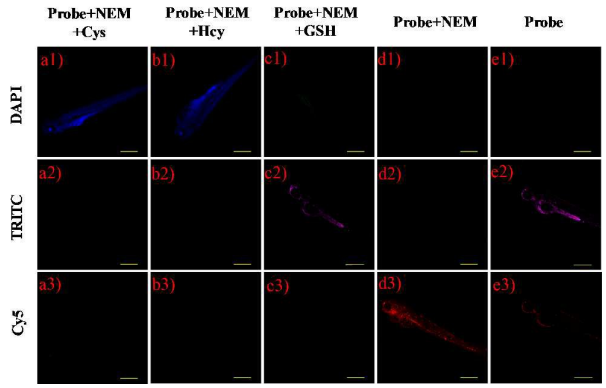


Fig. 5 One-photon fluorescence imaging of probe **CI** (5 μM) responding to respective biological thiols in zebrafish. a-c) The zebrafish were incubated with NEM (500 μM) for 30 min, followed by addition of Cys (200 μM), Hcy (200 μM) and GSH (200 μM) respectively for 15 min, then incubated with **CI** (5 μM) for 30 min and imaged; d) The zebrafish were incubated with NEM (500 μM) for 30 min, followed by addition of **CI** (5 μM) for 30 min and imaged. e) The zebrafish were incubated with **CI** (5 μM) for 30 min, then imaged. Scale bar = 500 μm.

Conclusions

In summary, we have developed a NIR fluorescent probe, named as CI, for efficiently detecting Cys/Hcy and GSH by multi-signal fluorescence mode. The probe showed some advantages, such as fast response, high selectivity and low cytotoxicity for biological system. The only probe displayed good fluorescent signal in the near infrared region. The probe **CI** could react with Cys/Hcy and GSH via a thiol-halogen SNAr nucleophilic substitution-rearrangement mechanism. The conjugated structure of **CI** was broken and released the classical TP coumarin dyes. And the two different fluorescent signals (blue and red) with two different excitation wavelengths were observed. the new probe could satisfy the need for imaging with the conversion of NIR to TP modes after detecting Cys/Hcy and GSH. Importantly, **CI** could sense Cys/Hcy and GSH in HeLa cells with OP and TP modes. Thus **CI** also achieved the sensing of Cys/Hcy and GSH in zebrafish with OP modes. The development of the new probe CI may open up a pathway for the detection of biological thiols, providing promising tools for revealing the interaction of biological thiols in various physiological and pathological conditions.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was financially supported by NSFC (21472067, 21672083), Taishan Scholar Foundation (TS 201511041), and the startup fund of the University of Jinan (309-10004).

Notes and references

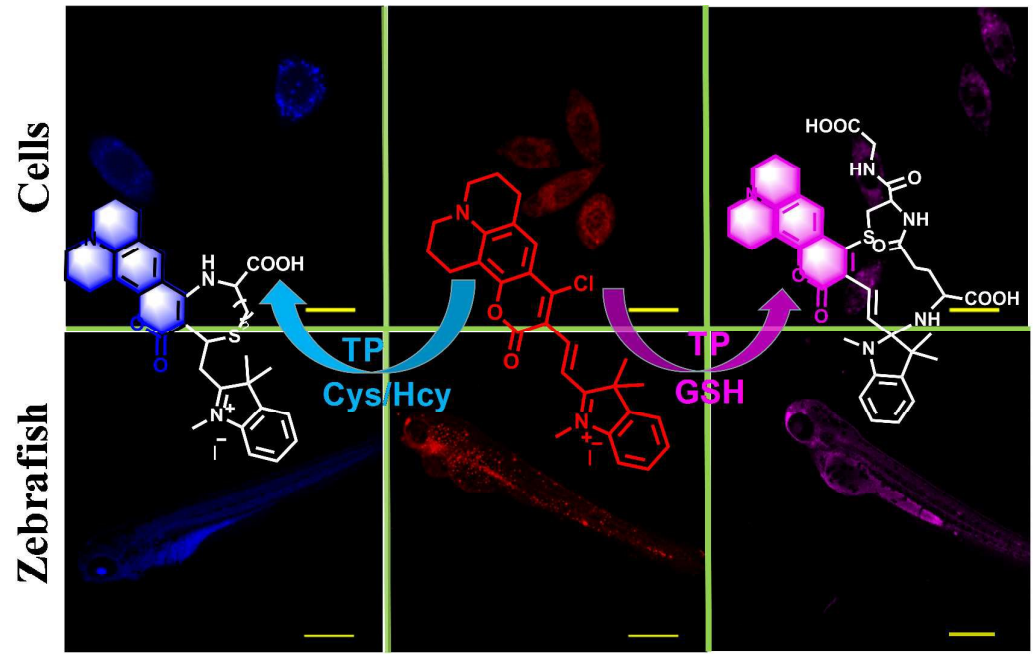
1 M. Kemp, Y. M. Go and D. P. Jones, *Free Radic. Biol. Med.,* 2008, **44**, 21-37.
2 G. I. Giles, K. M. Tasker and C. Jacob, *Free Radic. Biol. Med.*, 2001, **31**, 1279-1283.
3 J. R. Harris, *Trends Biochem. Sci.,* 2003, **28**, 32-40.
4 G. I. Giles and C. Jacob, *Biol. Chem.,* 2002, **383**, 375-388.
5 S. C. Lu, *Mol. Aspects Med.,* 2009, **30**, 95-116.
6 M. E. Anderson, *Chem-BiolInteract.,* 1998, **111**, 1-14.
7 D. M. Townsend, K. D. Tew and H. Tapiero, *Biomed. Pharmacother,* 2003, **57**, 145-155.
8 S. Shahrokhian, *Anal. Chem*., 2001, **73**, 5972-5978.
9 H. Refsum, P. M. Ueland, O. Nygård and S. E. Vollset, *Annu. Rev. Med.,* 1998, **49**, 31-62.
10 A. D. Smith, *Am. J. Clin. Nutr.,* 2002, **75**, 785-786.
11 D. G. Savage, J. Lindenbaum, S. P. Stabler and R. H. Allen, *Am. J. Med.*, 1994, **96**, 239-246.
12 J. G. Ray and C. A. Laskin, *Placenta,* 1999, **20**, 519-529.
13 G. G. Klee, *Clin. Chem.*, 2000, **46**, 1277-1283.
14 S. Sudha, B. Alexa, S. Jacob and F. J. Paul, *N. Engl. J. Med.*, 2002, **346**, 476-483.
15 O. Rusin, R. A. Agbaria, J. O. Escobedo, S. Jiang, I. M. Warner, F. B. Dawan, K. Lian and R. M. Strongin, *J. Am. Chem. Soc.*, 2004, **126**, 438-439.
16 L. Hu, S. Hu, L. Guo, T. Tang and M. Yang, *Anal. Methods*, 2016, **8**, 4903-4907.
17 J. S. Stamler and J. Loscalzo, *Anal. Chem.,* 1992, **64**, 779-785.
18 Y. Ogasawara, Y. Mukai, T. Togawa, T. Suzuki and S. Tanabe, *J. Chromatogr. B. Anal. Technol. Biomed. Life Sci.*, 2007, **845**, 157-163.
19 M. Rafii, R. Elango, G. Courtney-Martin, J. D. House, L. Fisher and P. B. Pencharz, *Anal. Biochem*., 2007, **371**, 71-81.
20 J. R. Lakowicz and C. Geddes, *Springer*, New York, 2005.
21 Y. Yang, Q. Zhao, W. Feng and F. Li, *Chem. Rev.*, 2013, **113**, 192-270.
22 Y. Tang, D. Lee, J. Wang, G. Li, J. Yu and W. Lin, *Chem. Soc. Rev*., 2015, **44**, 5003-5015.
23 B. Dong, X. Song, X. Kong, C. Wang, Y. Tang, Y. Liu and W. Lin. *Adv. Mater*., 2016, **28**, 8755-8759.
24 S. Zhu, W. Lin and L. Yuan, *Anal. Methods*. 2013, **5**, 3450-3453.
25 X. Cao, W. Lin and W. Wan, *Chem. Commun*., 2012, **48**, 6247-6249.
26 M. Göppert-Mayer, *Annalen Der Physik*, 1931, **401**, 273-294.
27 H. M. Kim and B. R. Cho, *Chem. Rev*., 2015, **115**, 5014-5055.
28 G. Carrone and R. Etchenique, *Anal. Chem.*, 2015, **87**, 4363-4369.
29 X. Chen, Y. Zhou, X. Peng and J. Yoon, *Chem. Soc. Rev.,* 2010, **41**, 2120-2135.
30 H. Chen, Y. Tang and W. Lin, *TrAC. Trends. Anal. Chem.*, 2016, **76**, 166-168.
31 A. Ah, G. Ur, F. Ali, N. Taye, S. Chattopadhyay and A. Das, *Chem. Commun*., 2015, **51**, 15592-15595.
32 H. Chen, Y. Tang, M. Ren and W. Lin, *Chem. Sci.*, 2016, **7**, 1896-1903.
33 C. Ge, H. Wang, B. Zhang, J. Yao, X. Lin, W. Feng, P. Zhou, Y.

View Article Online
DOI: 10.1039/C8NJ01793J

Wang and J. Fang, *Chem. Commun.*, 2015, **51**, 14913-14916.
34 X. Wang, J. Lv, X. Yao, Y. Li, Huang F, M. Li, J. Yang, X. Ruan and B. Tang, *Chem. Commun*., 2014, **50**, 15439-15442.
35 J. Zhang, J. Wang, J. Liu, L. Ning, X. Zhu, B. Yu, X. Zhang and H. Zhang, *Anal. Chem.*, 2015, **87**, 4856-4863.
36 H. Zhang, R. Liu, J. Liu, L. Li, P. Wang, S. Yao and H. Sun, *Chem. Sci.,* 2016, 7, 256-260.
37 Y. Kim, S. V. Mulay, M. Choi, S. Yu, S. Jon and D. G. Churchill, *Chem. Sci*., 2015, **6**, 5435-5439.
38 Y. Zhang, X. Shao, Y. Wang, F. Pan, R. Kang, F. Peng, Z. Huang, W. Zhang and W. Zhao, *Chem. Commun.,* 2015, **51**, 4245-4248.
39 Y. Tang, X. Kong, A. Xu and W. Lin, *Angew Chem. Int. Ed. Engl.*, 2016, **55**, 3356-3359.
40 P. Zhang, W. Liu, G. Niu, H. Xiao, J. Ge, J. We, H. Zhang, Y. Li and P. Wang, *J. Org. Chem.*, 2017, **82**, 3456-3462.
41 T. Gao, X. Cao, J. Dong, J. Liu, W. Lv, C. Li, X. Feng and W. Zeng, *Dyes Pigments,* 2017, **143**, 436-443.
42 H. Chen, B. Dong, Y. Tang and W. Lin, *Acc. Chem. Res.,* 2017, **50**, 1410-1422.
43 X. Dai, Z. Wang, Z. Du, J. Cui, J. Miao and B. Zhao, *Anal. Chim. Acta*., 2015, **900**, 103-110.
44 H. Li, W. Peng and W. Feng, *Chem. Commun.*, 2016, **52**, 4628-4631.
45 D. Li, Z. Wang and X. Cao, *Chem. Commun*., 2016, **52**, 2760-2763.
46 X. Feng, T. Zhang and J. Liu, *Chem. Commun.,* 2016, **52**, 3131-3134.
47 S. Liu, L. Zhang and T. Yang, *ACS Appl. Mater. Interfaces*, 2014, **6**, 11013-11017.
48 L. Niu, Y. Guan, Y. Chen, L. Wu, C. Tung and Q. Yang, *J. Am. Chem. Soc.* 2012, **134**, 18928-18931.

View Article Online
DOI: 10.1039/C8NJ01793J

Graphic content entry



A multi-signal fluorescent probe for discrimination of cysteine/homocysteine, and glutathione was engineered in living cells by one-photon and two-photon modes and zebrafish by one-photon modes.