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ORIGINAL ARTICLE

A New Endoplasmic Reticulum (ER)-Targeting Fluorescent Probe for the Imaging of Cysteine in Living Cells

Lei Zhou[1] · Yunxia Li[2] · Aiqin Zhou[3] · Guanghui Zhang[2] · Zhi-Qiang Cheng[1] · Yong-Xi Ge[1] · Shan-Kui Liu[1] · Ricardo Bentes Azevedo[4] · Juan Zhang[1] · Shenglin Jiang[2] · Cheng-Shi Jiang[1]

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Abstract

Cysteine (Cys) is an important endogenous amino acid and plays critical physiological roles in living systems. Herein, an endoplasmic reticulum (ER)-targeting fluorescent probe (**FER-Cys**) was designed and prepared for imaging of Cys in living cells. The probe **FER-Cys** consists of a fluorescein framework as the fluorescent platform, acrylate group as the response site for the selective recognition of Cys, and ER-specific *p*-toluenesulfonamide fragment. After the response of probe **FER-Cys** to Cys, a turn-on fluorescence signal at 546 nm could be detected obviously. The probe **FER-Cys** further shows desirable selectivity to Cys. Finally, the probe **FER-Cys** was proven to selectively detect Cys in live cells and successfully image the changes of Cys level in the cell models of H_2O_2-induced redox imbalance.

Keywords Imaging of cysteine · Endoplasmic reticulum-targeting · Fluorescent probe · Selective recognition · Redox imbalance

Introduction

As a prevalent endogenous amino acid, cysteine (Cys) could be ubiquitously originated de novo in living systems under normal physiological conditions [1]. Cys exhibits remarkable reductibility because of the presence of thiol group which is a well-known reductive group, and participates in many physiological processes such as reducing dopaquinone to inhibit the production of melanin, scavenging reactive oxygen species (ROS) during ER stress. Cys takes part in a variety of enzymatic reactions and displays many critical biological roles, such as ion-sulfur binding, acting as precursor to the antioxidant glutathione (GSH), and protein synthesis [2–6]. The shortage of Cys in body can block the biosynthesis of tripeptide, and result in the risk of various diseases including cancer, hair depigmentation, liver damage, lethargy, neuropsychiatric and immune dysfunctions [7–9]. It's reported that the rapid and precise detection of the Cys concentration could be used to monitor vitamin B deficiency, inflammatory conditions and metabolic syndrome [10, 11]. Therefore, rapid and precise detection of the Cys level in living systems is of great importance to study its biological roles.

Endoplasmic reticulum (ER) is an important organelle in eukaryotic cells, and plays a crowd of crucial biological functions particularly in protein secretion and folding, lipid biosynthesis, and calcium homeostasis [12, 13]. When ER was stimulated by various cellular disturbances, ER stress occurs and has been involved in many diseases such as cancer, Parkinson's disease, and Alzheimer's disease [14–16]. Abnormal levels of reactive oxygen species (ROS) could cause ER stress, and Cys has been proved as a vital reductive thiol to mediate redox state of ER by scavenging ROS during ER stress [17, 18]. However, the high level of Cys in body is

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✉ Juan Zhang
bio_zhangj@ujn.edu.cn

✉ Shenglin Jiang
jsl@laiyu.com

✉ Cheng-Shi Jiang
bio_jiangcs@ujn.edu.cn

[1] School of Biological Science and Technology, University of Jinan, Jinan 250022, China

[2] Laiyu Chemical Co., Itd, Laizhou, China

[3] College of Horticulture, Qingdao Agricultural University, Qingdao 266109, China

[4] Institute of Biological Sciences, University of Brasília, Brasilia 70910900, Brazil

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toxic mostly because of the abnormally production of homocysteine [19]. In addition, oxidation of Cys by Cys dioxygenase could lead to the formation of taurine and sulfate, which probably exert a favorable effect on acid-base balance in these animals who received excess sulfur amino acid without an overall increase in amino acids [19]. Meanwhile, the excessive Cys could give rise to the extensive vacuoles in cytoplasm and trigger ER stress.[12] Thus, specific detecting the level of Cys in ER is highly important for the in-depth study of its physiological roles.

Traditionally, the methods used to detect Cys mainly include spectrophotometry, liquid chromatography, fluorescence spectroscopy, and colorimeteric methods [20–28]. However, these methods generally cannot achieve nondestructive testing and in situ testing of samples, and are not suitable for the rapid and in situ detection of cellular Cys. Currently, fluorescent imaging is an appealing technique for the detection of biomolecules due to the high sensitivity, real-time and nondestructive imaging manner [29–31]. To date, lots of fluorescent probes for monitoring cellular Cys level have been developed [32, 33]. However, these generally are not ER-targeting probes, and lack selectivity in the detection of Cys in ER. Currently, the ER-targeting fluorescent probe for the imaging of Cys is still very limited, and therefore fabrication of ER-targeting fluorescent probes for detection of Cys in living systems is still in urgent need.

Against this background, we herein present a new ER-targeting fluorescent probe (**FER-Cys**) for the imaging of Cys in living cells. The probe **FER-Cys** used a fluorescein derivative as the fluorescent platform due to the eminent optical advantages like the high fluorescence quantum yield, and utilized acrylate group as the response site for the selective recognition of Cys. Meanwhile, *p*-toluenesulfonamide was selected to make the probe ER-targeting ability. After the response of probe **FER-Cys** to Cys, an obviously increased fluorescence signal at 546 nm could be observed. In addition, probe **FER-Cys** showed marked selective response to Cys over the other thiols and some prevalent ions in living systems. Moreover, this probe **FER-Cys** can be successfully applied in the selective detection of endogenous Cys in living cells, and image the changes of Cys level in the cell models of H_2O_2-induced redox imbalance.

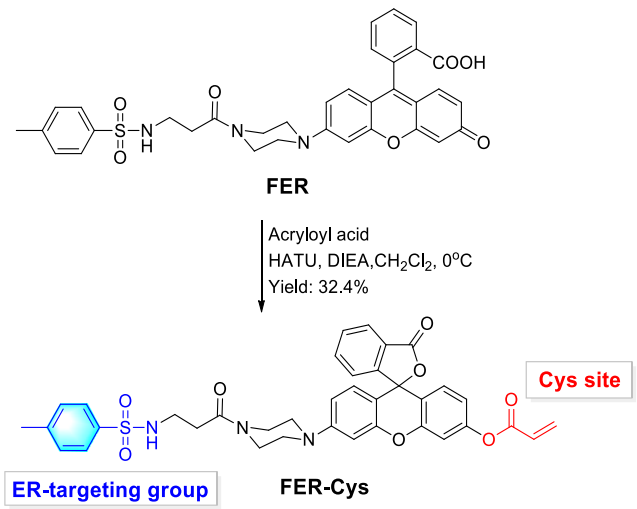
Experimental

Materials and Instruments

All solvents and reagents were purchase from Shanghai Aladdin Co. Ltd., and used without further purification. TLC analysis was conducted on Yantai JingYou GF_{254} silica gel plates, and flash column chromatography was performed on Isolera One system (Biotage, Uppsala, Sweden) using silica gel (mesh 200–300, Qingdao Hailang Inc). ^1H and ^{13}C NMR spectra were recorded on Bruker Avance III 600 MHz NMR Spectrometer, using solvent signal ($CDCl_3$, δ_H 7.26 and δ_C 77.2 ppm) as internal reference. High-resolution mass spectra (HRMS) were collected using Agilent 6520 Q-TOF LC/MS. PBS buffer (20 mM, 5% MeOH, pH = 7.4) was used. UV/vis spectra were recorded using a Shimadzu UV-2700 spectrophotometer, and fluorescence spectra and relative fluorescence intensity were measured on a Hitachi F4600 spectrofluorimeter. Cells imaging was made using a Nikon A1R confocal microscope.

Synthesis of Compound FER-Cys

Scheme 1 shows the synthetic route of **FER-Cys**. The target compound **FER-Cys** was synthesized from the starting compound **FER**, which was prepared according to our previously reported protocol [31]. Briefly, the solution of acrylic acid (34 μL, 0.5 mmol) dissolved in dry dichloromethane (2.5 mL) was slowly added into the dichloromethane solution (2.5 mL) containing **FER** (62.6 mg, 0.1 mmol), 2-(7-Azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HATU, 49.4 mg, 0.13 mmol) and N-diisopropylethylamine (DIEA, 100 μL) at 0 °C. The mixture was stirred for 30 min, and then kept stirring overnight at room temperature. The solvent was removed in vacuo to obtain a crude mixture, which was further extracted with dichloromethane (3 × 10 mL). The organic phase was dried over Na_2SO_4, and concentrated in vacuo. The resulting residues was subjected to the flash column chromatograph with eluent of CH_3OH/dichloromethane (1/60, *v*/v) to obtain product **FER-Cys** (22 mg, 32.4%). ^1H NMR (600 MHz, $CDCl_3$) δ 8.02 (d, J = 7.6 Hz, 1H), 7.74 (d, J = 8.1 Hz, 2H), 7.67 (dd, J = 7.6 7.4 Hz, 1H), 7.62 (dd, J = 7.6 7.4 Hz, 1H), 7.29 (d, J =



Scheme 1 Synthetic route of the probe **FER-Cys**

8.1 Hz, 2H), 7.17 (d, J = 7.6 Hz, 1H), 7.11 (d, J = 1.8 Hz, 1H), 6.83–6.79 (m, 2H), 6.66–6.70 (m, 2H), 6.64–6.57 (m, 2H), 6.34–6.28 (m, 1H), 6.05 (d, J = 10.5 Hz, 1H), 5.56 (t, J = 6.6 Hz, 1H, NH), 3.73–3.67 (m, 2H), 3.54–3.49 (m, 2H), 3.24–3.16 (m, 6H), 2.58 (t, J = 5.5 Hz, 2H), 2.40 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.7, 169.4, 164.1, 153.0, 152.4, 152.3, 152.1, 151.9, 143.4, 137.2, 135.2, 133.4, 129.9, 129.8, 129.2, 129.0, 127.6, 127.1, 126.7, 125.2, 124.1, 117.4, 116.9, 112.5, 110.4, 109.8, 102.6, 82.7, 48.3, 48.1, 44.8, 41.2, 39.2, 33.0, 21.6. HR-ESIMS: $[M+H]^+$ calcd for $C_{37}H_{34}N_3O_8S^+$ 680.2061, found 680.2052.

Photophysical Analyses

For the UV-Vis absorption detection: The UV-vis absorption spectrum of the probe **FER-Cys** (5 μM) in PBS solution (20 mM, 5% MeOH, pH 7.4), was measured using a Shimadzu UV-2700 spectrophotometer. After the addition of Cys (50 or 100 μM), the UV-vis absorption spectrum of the probe **FER-Cys** (5 μM) in PBS solution (20 mM, 5% MeOH, pH 7.4) was also recorded.

For the fluorescence detection: The fluorescence measurements of the solutions were carried out using a Hitachi F4600 spectrofluorimeter at room temperature. The fluorescence emission spectra of the solutions from 510 to 650 nm were obtained under excitation at 500 nm with the slits set at 5.0/5.0 nm. Before measuring the fluorescence data, the probe **FER-Cys** (5 μM) in PBS solution (20 mM, 5% MeOH, pH 7.4) was treated with various concentrations of Cys (0–200 μM) for 30 min. The selectivity of the probe **FER-Cys** toward Cys was measured by comparison with other possible competitive species. The probe **FER-Cys** (5 μM) was dissolved in PBS solution was treated with Cys or other possible competing species for 30 min, and the excitation wavelength was 500 nm. Time-dependent fluorescence spectra 5 μM **FER-Cys** in presence of 100 μM Cys were recorded under excitation at 500 nm in PBS (20 mM, 5% MeOH, pH = 7.4). Fluorescence spectra of 5 μM **FER-Cys** at various pH were measured under excitation at 500 nm in PBS (20 mM, 5% MeOH, pH = 7.4).

Cytotoxicity Bioassay

The in vitro cytotoxicity of **FER-Cys** was evaluated according to the MTT protocol. HeLa cells were seeded into 96 well-plates at 1×10^4 cells/well and allowed to attach for 24 h. Then, different concentrations of **FER-Cys** were added into wells for co-culture with cells for 24 h. Then, 10 μL of MTT (5 μg/mL in PBS) were mixed into each cells to incubate for another 4 h. Then, supernatants were aspirated, and 100 μL of dimethyl sulfoxide (DMSO) were used to solve the formed formazan. Finally, the absorbance was determined at 490 nm using a Microplate Reader (Tecan, Switzerland).

Cells Culture and Fluorescence Imaging

HeLa cells were cultured for 24 h in the glass-bottom culture dishes. For the experiment to image endogenous Cys, the Hela cells were co-incubated with 10 μM **FER-Cys** for 20 min at 37 °C, and then the media was replaced with PBS buffer. For the experiment of imaging exogenous Cys, the Hela cells pre-treated with *N*-ethylmaleimide for 0.5 h were incubated with 10 μM **FER-Cys** for 0.5 h. Then each well was added 100 μM **Cys** and the plates were incubated for another 20 min. All the cell imaging was acquired in a Nikon A1 confocal microscope.

Results and Discussion

Design and Synthesis of the Probe FER-Cys

The probe **FER-Cys** was rationally designed based on the following analyses. Firstly, fluorescein-based derivatives are the highly useful fluorescent platforms for the bio-imaging due to the excellent optical advantages including high fluorescence quantum yield and desirable photostability. Therefore, in this work a fluorescein derivative as the platform was selected to construct the fluorescent probe. Secondly, acrylate is a well-known response site for the selective recognition of Cys, and thus was utilized for the development of Cys probe [34]. Finally, ER-targeting *p*-toluenesulfonamide was attached on the fluorescein-based fluorophore to make the probe **FER-Cys** possess selective ER-targeting property. Scheme 1 shows the synthetic route of the probe **FER-Cys**, and its

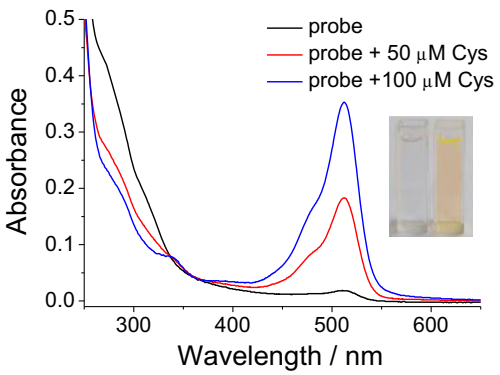


Fig. 1 UV-Vis absorption spectra of 5 μM **FER-Cys** treated with or without 50 or 100 μM Cys in PBS (20 mM, 5% MeOH, pH 7.4). Inset: Image of 5 μM **FER-Cys** in absence (right) and presence (left) of 100 μM Cys

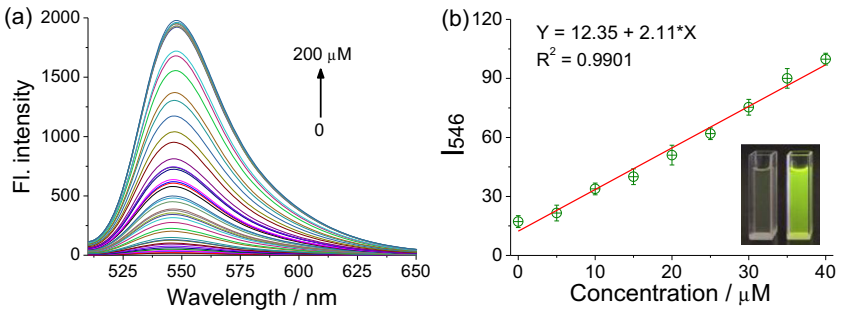


Fig. 2 (**a**) Fluorescence spectra of 5 μM **FER-Cys** upon the addition of 0–200 μM Cys in PBS buffer (20 mM, 5% MeOH, pH = 7.4) under the excitation at 500 nm. (**b**) Fluorescence intensity at 546 (I_{546}) nm as a function of Cys concentration. Inset: Fluorescent image of 5 μM **FER-Cys** treated with (right) or without (left) 100 μM Cys under the irradiation of a UV lamp

structure was confirmed by ^{1}H NMR, ^{13}C NMR and HR-ESIMS data (Supporting Information).

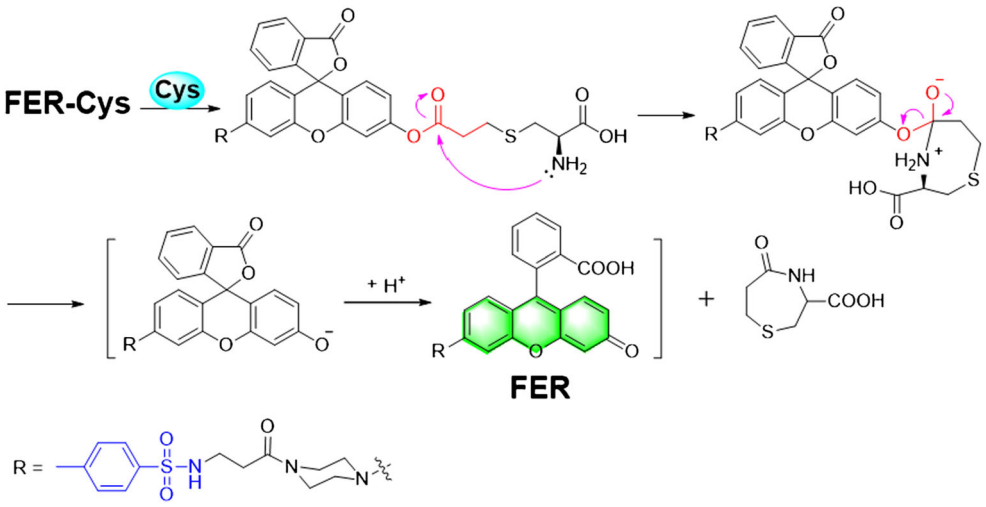
Optical Response of FER-Cys to Cys

To explore the optical response behaviour of the probe **FER-Cys** to Cys, UV-Vis absorption spectra of probe **FER-Cys** treated with or without Cys were tested. As shown in Fig. 1, before the treatment with Cys the probe **FER-Cys** itself displayed nearly no absorption in the visible region, due to that the fluorescein fluorophore possessing spirolactone structure could keep ring-closing form in PBS. After the treatment of **FER-Cys** with 50 μM or 100 μM Cys, an obvious absorption band at 510 nm was observed, indicating that the fluorescein fluorophore was transformed into ring-opening form. Correspondingly, the colour of the solution significantly changed from colourless to yellow within minutes after the response of the probe to 100 μM Cys (Fig. 1).

Next, the fluorescence spectra of probe **FER-Cys** in absence and presence of Cys were measured to assess its fluorescence response property to Cys. The free **FER-Cys** hardly displayed fluorescence with the fluorescence quantum yield (Φ) of 0.002 under the excitation at 500 nm. Whereas the treatment of probe **FER-Cys** with Cys could lead to the obviously increased fluorescence at 546 nm, meanwhile, the fluorescence intensity at 546 nm (I_{546}) increased progressively along as Cys concentrations enlarging from 0 to 200 μM (Fig. 2a). Additionally, linear correlation between the fluorescence intensity at 546 nm and the Cys level (0–40 μM, R = 0.9901) could be obtained, and according to the IUPAC recommendations the detection limit was evaluated to be 0.21 μM (S/*N* = 3) (Fig. 2b). Correspondingly, the fluorescent colour of **FER-Cys** in PBS buffer significantly changed from a weak green to a strong green after the reaction of the probe to 100 μM Cys. Therefore, the probe **FER-Cys** could function as a sensitive fluorescent probe for Cys.

The mechanism for the response of probe **FER-Cys** to Cys was then probed by HRMS assay. Based on that the α,β-unsaturated carbonyl (Michael Acceptor) could conjugate with Cys to provide 7-membered ring thioether [35], we speculated that the probe **FER-Cys** could reacted with Cys by the addition reaction at first, and then the amine group attacked the carbonyl to dissociate the dye **FER** and 7-membered thioether (Scheme 2). In addition, the HRMS assay of the

Scheme 2 Proposed response mechanism of FER-Cys towards Cys



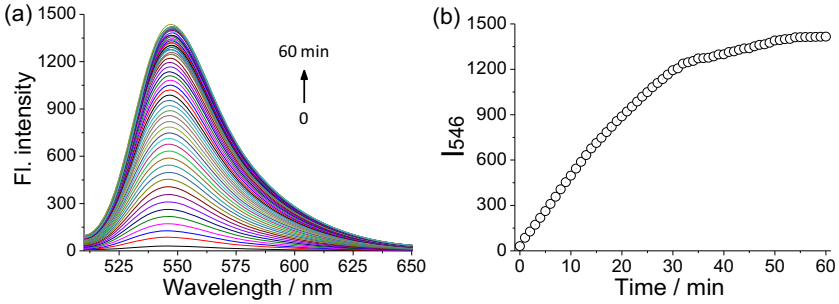


Fig. 3 Time-dependent fluorescence spectra and intensities at 546 nm of 5 μM **FER-Cys** in presence of 100 μM Cys under excitation at 500 nm in PBS (20 mM, 5% MeOH, pH = 7.4)

response products of **FER-Cys** to Cys (Fig. S2) obviously displayed that two peaks at *m/z* 626.1960 corresponding to the dye **FER** and thioether.

The time-dependent fluorescence spectra of probe **FER-Cys** treated with 100 μM Cys was tested. When 100 μM Cys was added to the solution of 5 μM **FER-Cys** in PBS buffer, it was shown that the fluorescence intensity at 546 nm continuously increased in a time-dependent manner, with maximum reaching at about 30 min (Fig. 3). Thus, probe **FER-Cys** could potentially serve as a sensitive fluorescent probe for imaging Cys.

Subsequently, the selectivity of probe **FER-Cys** towards Cys was explored by the fluorescence chromatography. **FER-Cys** was treated with the other twenty-one biologically related species including GSH, ClO^- and S^{2-}. As shown in Fig. 4, an obvious enhanced fluorescence at 546 nm (> 60-fold) could be observed after the treatment of **FER-Cys** with Cys. However, the fluorescence spectra of probe **FER-Cys** exhibited no remarkable change after the addition of the other species. Therefore, the probe **FER-Cys** displayed desirable reaction selectivity towards Cys.

The pH effect on the fluorescent response of probe **FER-Cys** towards Cys was evaluated. The result was displayed in Fig. S1. The free probe **FER-Cys** displayed very weak fluorescence in the pH range 4.0–9.5. However, after the reaction of probe with Cys, the fluorescence intensity at 546 nm was significantly enhanced in the pH range 6.3–9.5. Due to the desirable fluorescence response of probe **FER-Cys** to Cys at normal physiological pH of about 7.4, the probe **FER-Cys** could be used in the imaging of cellular Cys.

Fluorescence Imaging in Living Cells

To probe the feasibility of probe **FER-Cys** to image endogenous Cys in living cells, the fluorescent response of probe **FER-Cys** towards Cys in Hela cess was tested. Initially, the cytotoxicity of the probe **FER-Cys** was evaluated by MTT assay to obtain the safety dosage range. The bioassay results in Fig. S3 verified that the probe **FER-Cys** showed low toxicity towards HeLa cells under the concentration of 0–10 μM. After treated with 5 μM **FER-Cys** for 30 min, the HeLa cells showed strong green fluorescence under excitation at 561 nm. After the pre-treatment with 1 mM *N*-ethylmaleimide (NEM) for 30 min and then incubation with 5 μM **FER-Cys** for another 30 min, the HeLa cells displayed nearly no fluorescence due to the fact that NEM eliminated the cellular thiols including Cys. However, the strong green fluorescence in HeLa cells appeared again after the further treatment of HeLa cells with 100 μM Cys for 30 min (Fig. 5). This result indicates the good ability of probe **FER-Cys** to make response to endogenous and exogenous Cys in living cells.

Next, the capability of probe **FER-Cys** to selectively detect Cys in ER was explored. The HeLa cells were incubated with 5 μM **FER-Cys** for 30 min, and then treated with 1 μM ER-Tracker Blue. The distribution of **FER-Cys** in Hela cells was determined by the colocalization experiments with commercial ER-specific dyes. From the results in Fig. 6, the green fluorescence in treated cells presented desirable overlap with the blue fluorescence from ER-tracker Blue, and the Pearson's colocalization coeffcient (R) value of green and blue

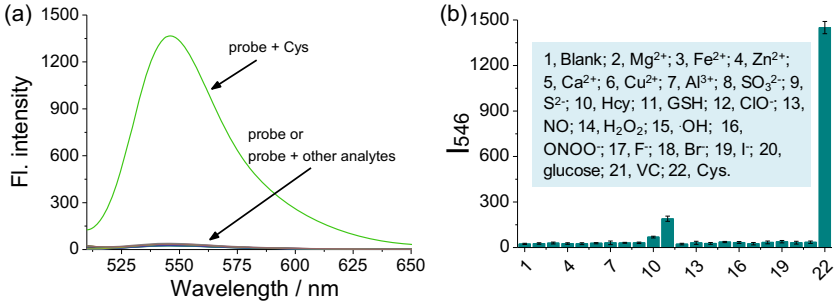


Fig. 4 Fluorescence spectra (**a**) and fluorescence intensity at 546 nm (**b**) of 5 μM **FER-Cys** in presence of various species dissolved in PBS (20 mM, 5% MeOH, pH = 7.4) under excitation at 500 nm. Concentration: GSH, 1 mM; the other species, 50 μM

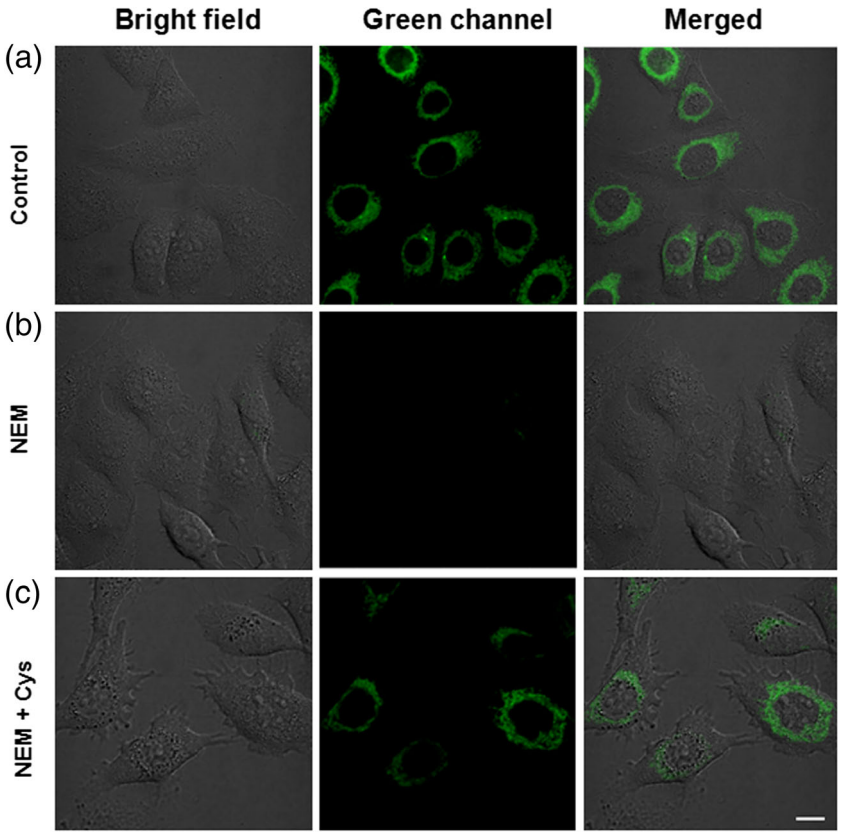


Fig. 5 Fluorescent images of HeLa cells. **a**: HeLa cells were treated with 5 μM **FER-Cys** for 30 min. **b**: HeLa cells were pretreated with 1 mM *N*-ethylmaleimide (NEM) for 30 min and then incubated with 5 μM **FER-Cys** for 30 min. **c**: HeLa cells pretreated with 1 mM NEM for 30 min was incubated with 5 μM **FER-Cys** for 30 min, and then treated with 100 μM Cys for another 30 min. Emission was collected at 570–620 nm with excitation at 561 nm. Scale bar: 10 μm

fluorescence was calculated to be 0.95. This result indicated that the probe **FER-Cys** was manily distributed in ER, demonstrating **FER-Cys** could be taken as an specific fluorescencet probe for Cys in ER.

Redox imbalance generally occurs when the excess ROS produces or the protective antioxidants decrease, and is closely related with inflammation and neurodegenerative diseases [36, 37]. Cys is a prevalent antioxidant playing important roles for the maintenance of cellular redox balance [38, 39]. Herein, we explored the responsive capability of probe **FER-Cys** to the changes of the endogenous Cys level by changing the cellular redox balance with H_2O_2 (one of main types of ROS). The results in Fig. 7 showed the HeLa cells incubated with 5 μM **FER-Cys** having strong green fluorescence that could be attributed to the reaction of probe **FER-Cys** with endogenous Cys. However, when HeLa cells were pretreated with 200 μM or 400 μM H_2O_2 and then stained with 5 μM **FER-Cys** for 30 min, it can be found that the green fluorescence decreased slightly (Fig. 7b and c). Based on these above results, it was proved that probe **FER-Cys** could be taken as a potential tool molecule to image the Cys level changes in living cells.

Conclusions

In conclusion, a new sensitive and selective far-red fluorescent probe **FER-Cys** for detecting Cys in living cells was discovered in the present work. The probe **FER-Cys** was built by an ER-targeting fluorescein derivative **FER** coupling with acrylate group as the response site for the selective recognition of Cys. In its response to Cys, a turn-on fluorescence signal at

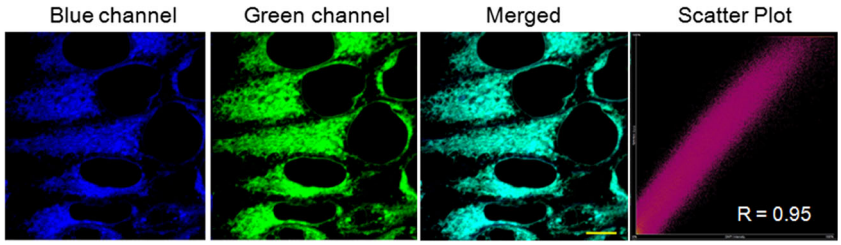


Fig. 6 Images of HeLa cells costained with 5 μM **FER-Cys** for 30 min, and then treated with 1 μM ER-tracker red for 10 min, respectively. Blue channel (425–475 nm), λex = 405 nm; Green channel (570–620 nm), λex = 561 nm. Scale bar = 10 μm

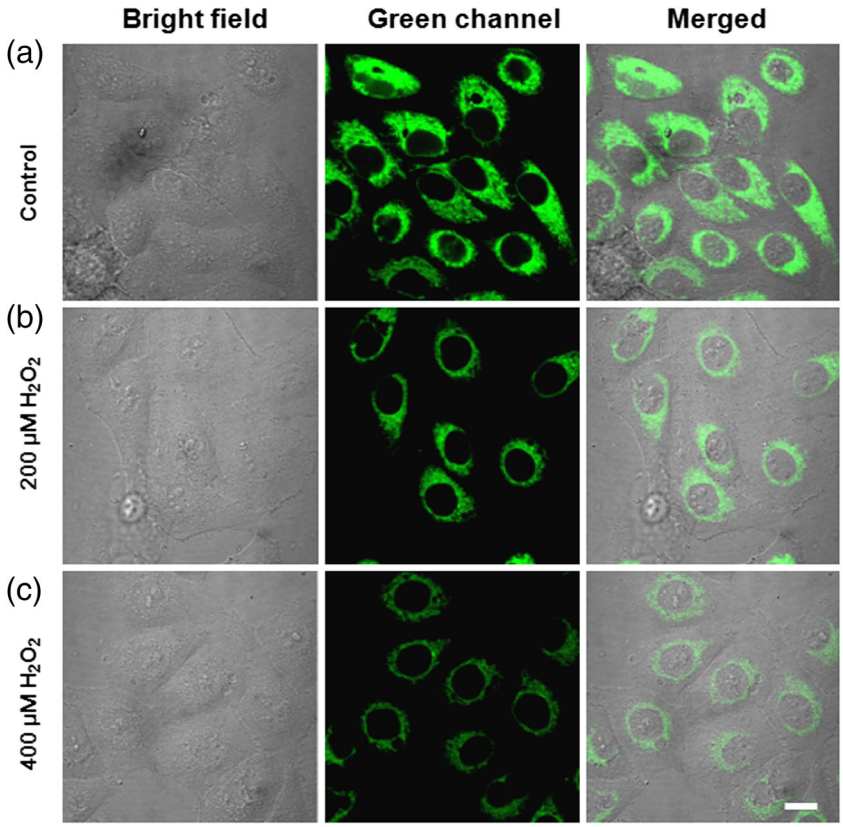


Fig. 7 (**a**) HeLa cells treated with 5 μM **FER-Cys** for 30 min. (**b**) HeLa cells pretreated with 200 μM H_2O_2 for 30 min and then treated with 5 μM **FER-Cys** for 30 min. (**c**) HeLa cells pretreated with 400 μM H_2O_2 for 30 min and then treated with 5 μM **FER-Cys** for 30 min. Green channel (570–620 nm), λ_{ex} = 405 nm. Scale bar = 10 μm

546 nm could be observed obviously. In addition, the probe **FER-Cys** showed high selectivity to Cys over twenty-one tested species. The biological applications demonstrated that probe **FER-Cys** could be applied to selectively image endogenous Cys in live cells, and detect the changes of Cys level in the cell models of H_2O_2-induced redox imbalance.

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Compliance with Ethical Standards

Conflict of Interest There are no conflicts to declare.

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