A novel coumarinocoumarin-based two-photon fluorescent cysteine biosensor for targeting lysosome

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A novel coumarinocoumarin-based two-photon fluorescent cysteine biosensor for targeting lysosome

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ABSTRACT: Coumarinocoumarin, one of the coumarin derivatives, is easy to synthesize and have rich modification sites. The large conjugate plane of coumarinocoumarin make it has more excellent optical property than conventional coumarin, for example, the two-photon fluorescence property. So, the coumarinocoumarin-based probe (**CCy**) has been designed and synthesized, which is the first lysosomal targeting fluorescent biosensor for cysteine. This probe was prepared by a 3-step procedure as a latent fluorescence probe to achieve high sensitivity and fluorescence turn-on response toward cysteine (Cys) over homocysteine (Hcy), glutathione (GSH) and other various natural amino acids under physiological conditions. Upon addition of Cys to the solution of **CCy**, remarkable enhancement on 520 nm of fluorescence spectra can be monitored. This probe was then successfully used for fluorescence imaging of Cys in mice organ tissues and HeLa cells, and quantitative determination had been achieved within a certain range, which proved the permeability of **CCy**. The concentration of Cys in animal serum was measured and the viability exceeded 80%, indicating that **CCy** can be a biocompatible and rapid probe for Cys in vivo. Simultaneously, its ability to detect Cys in lysosome has been validated by its lysosomal targeting.

INTRODUCTION

A myriad of bioprocesses are related to biothiols such as Cys, Hcy, and GSH. Cys and Hcy are involved in cellular growth, while GSH plays an important role in redox homeostasis. For example, Cys is the only amino acid with a thiol functional group that serves as a unique unit in protein construction, enzyme active sites and cofactors.[1-3] Changes in cell thiol levels are critical in the development of many diseases. Deficiency of biothiols can lead to various complications, such as substance abuse, lethargy, liver damage, psoriasis and slow growth in children. While the high level of biothiols in blood is considered to be associated with Alzheimer's disease, cardiovascular, osteoporosis, cancer, inflammatory bowel disease and AIDS.[4-6] Despite the similar structures of Cys, Hcy and GSH, they play different important biological roles, associated with different diseases. Therefore, the detection and discrimination of thiol-containing molecules in biological samples are of great importance.

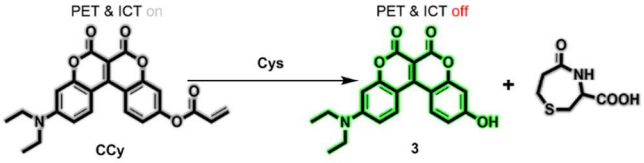
So far, a variety of strategies, include mass spectrometry, high performance liquid chromatography (HPLC), capillary electrophoresis, optical assay, and the electrochemical method, have been developed to detect thiols.[7-12] While these methods provide high accuracy, but required certain skill level, expensive analytical instruments and cumbersome preconditioning procedures. All that makes it unsuitable for on-site trials and quick detection. In addition, due to the limitations of in vivo studies, these methods cannot be applied to intracellular assays. Among the available techniques to detect and quantify thiols, optical assay based on fluorescent probe has been generally affirmed since they are convenient to operate, sensitive, selective, and can detect intracellular mercapto biomolecules.

In the past decade, various fluorescent probes for thiols based on different mechanisms have been studied. These mechanisms include thiol cleavage reaction of sulfonamide and sulfonate esters[13,14], cyclization reaction with aldehyde[15,16], Michael addition[17-19], thiol-halogen nucleophilic substitution[20], disulfide exchange reaction[21-23], native chemical ligation (NCL) mechanism[24,25], metal complexes-displace coordination and others[26-28]. Among the various strategies, conjugate addition-cyclization reaction with Cys/Hcy just received little attention[29-31]. It has been known that the conjugate addition of Cys to acrylates will generate the corresponding thioether, which can further undergo an intramolecular cyclization to yield 3-carboxy-5-oxoperhydro-1, 4-thiazepine. Meanwhile, the cyclization of Hcy is not stable, and GSH can only occur addition reaction. Many previous work has shown that the acryloyl group have good selectivity towards Cys[32-34]. Simultaneously, acryloyl is selected not only because the electrophilic but also can cause fluorescence quenching. Acryloyl as an electron acceptor for the Photoinduced Electron Transfer (PET) process, leading to a low fluorescence of probe. Acryloyl causes the electron-donating ability of hydroxyl decreased, so the emission of coumarinocoumarin redshift from 520 nm to 550 nm, which due to the Intramolecular Charge Transfer (ICT) effect. After treatment with thiols, corresponding thioethers are formed, and the disappearance of PET effect can make the fluorescence recovery. Simultaneously, because of the decrease of ICT effect, emission blue-shift from 550 nm to 520 nm.

Coumarin was selected as the fluorophore due to its wide bioactivities (antioxidant, anti-inflammatory, anticancer, MAO-B inhibitory and antimicrobial activities) and desirable photophysical properties (visible emission wavelengths, large Stokes' shift and high fluorescence quantum yields[35-38]. In addition to the exploration on the derivative of single coumarin, coumarinocoumarin exhibits more advantages such as red shift in excitation and emission wavelength and larger Stocks shift. The fused heterocycle is beneficial for transferring electrons, and the abundance of electrons in coumarin-fused heterocycles implies that the coumarinocoumarin must be excellent fluorophore.[39,40] Most of all, due to the large conjugated planar, coumarinocoumarin bear two-photon excitation property.

Lysosome is an important cell organelle that contains approximately 50 different degradative enzymes which are active at the acidic pH. Herein, there are considerable amount of Cys, GSH and Hcy which convert from methionine in the lysosome. Moreover, such as Cys, thiols facilitate intralysosomal proteolysis by reducing disulphide bonds. The necessary molecular decoration for lysosome targeting was incorporated via the N, N-diethyl group but not the morpholine ring. Because the lysosome targeting dyes like neutral red and acridine orange catch N, N-dimethyl group, and it's not necessary to waste a site to connect morpholine ring.

Based on the abovementioned reason and our earlier works[41,42], a coumarinocoumarin-based probe (**CCy**) bearing acrylate moieties for selective recognition of Cys over Hcy and GSH was made, and which is the first lysosomal targeting fluorescent biosensor for specifically detect Cys[43-46]. The application of **CCy** in cellular and vivo was researched, meanwhile, its two-photon excitation properties and the application in bioimaging was measured.



Scheme 1. Design concept of **CCy** for Cys.

EXPERIMENTAL SECTION

Materials and instruments. Commercial available compounds were used without further purification. Fluorescence spectra were carried out a Hitachi F-7000 luminescence spectrometer. UV–visible spectra were recorded with a Varian Cary 100 spectrophotometer. NMR spectra were recorded on a JEOL ECS 400M spectrometer and chemical shifts were referenced relative to tetramethylsilane. Mass data (ESI) were obtained by a LQC system (Finnegan MAT, USA). The melting points were measured with an X-6 melting point apparatus. Stock solutions (2 mM) of amino acids were prepared in ultrapure water, and stock solution of **CCy** (2×10^{-3} M) was prepared in DMSO. For all fluorescence measurements, test samples were obtained by injecting 2 μL of the probe stock solution into 2 mL PBS buffer. After addition of analytes for 10 min at 20 °C, fluorescence spectra and UV–visible spectra were measured. An excitation and emission slit widths of 5.0 nm were used for all fluorescent measurements.

Cell and tissue culture. All the cells in this paper are HeLa cells. Hela cells were maintained at humidified atmosphere at 37℃ incubator. MTT assay was used to determine the cytotoxic effect. The animal serums were from Guangzhou Hongquan biotechnology co. LTD. The tissue experiments used 6-8 weeks old Kunming mice (18-22 gram). LysoTracker Red is DND 99 from Thermo Fisher. MitoTracker Red is CM-H2XRos from Thermo Fisher.

Synthesis. Compound **2**, **3** and **CCy** were synthesized according to the methods described in supporting information (Scheme S-1). The property of **CCy**: m.p. 132 °C. MS (*m/z*, ESI) *m/z* = 406.1704 [M + H] (Fig. S-25). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.39 (d, J = 9.0 Hz, 1H), 8.14 (d, J = 9.5 Hz, 1H), 7.42 (d, J = 2.4 Hz, 1H), 7.32 (dd, J = 8.9, 2.4 Hz, 1H), 6.88 (dd, J = 9.5, 2.6 Hz, 1H), 6.64 (d, J = 4.5 Hz, 1H), 6.59 (s, 1H), 6.47 (dd, J = 17.2, 10.3 Hz, 1H), 6.24 (d, J = 10.3 Hz, 1H), 3.53 (q, J = 7.1 Hz, 4H), 1.18 (t, J = 7.0 Hz, 6H).

RESULTS AND DISCUSSION

UV-vis absorption and fluorescence spectra. The sensing properties of **CCy** for thiols were primarily investigated. The absorption and fluorescence emission spectra of **CCy** (2 μM) in PBS buffer (20 mM, pH 7.4) are shown in Fig. 1. As expected, the probe is nearly non-fluorescence under excitation at 445 nm since the influence of PET effect caused by acryloyl group. After its treatment with Cys (40 μM) for 10 min at room temperature (20 °C), **CCy** exhibited a reduction of absorption intensity at 445 nm, and meanwhile, the fluorescence emission peak at 550 nm blue-shifted to 520 nm and the fluorescence intensity at 520 nm has a significantly increase.

In order to study the reaction kinetics, time-dependent fluorescence spectra change of **CCy** with thiols were evaluated. **CCy** (2 μM) was treated with 40 μM of Cys, Hcy or GSH, and then the fluorescence intensity enhancement at 520 nm was recorded (Fig. 1c). It's obvious that the fluorescence intensity of the sample treated with Cys is always stronger than the one treated with Hcy or GSH. All these indicate that **CCy** can serve as an excellent fluorescent probe for rapidly separate Cys with Hcy and GSH.

The emission intensity shows a clearly increase in the fluorescence spectra of **CCy** when the concentration of Cys increases (Fig. 1d). Meanwhile, a good linearity (R^2 = 0.995) between the fluorescence intensity at 520 nm and the Cys concentration in the range of 0-20 μM can be observed (Fig. S-6). Moreover, the detection limit of **CCy** for Cys was calculated to be 0.411 μM, indicating that **CCy** can serve as a sensitive quantitative Cys detector.

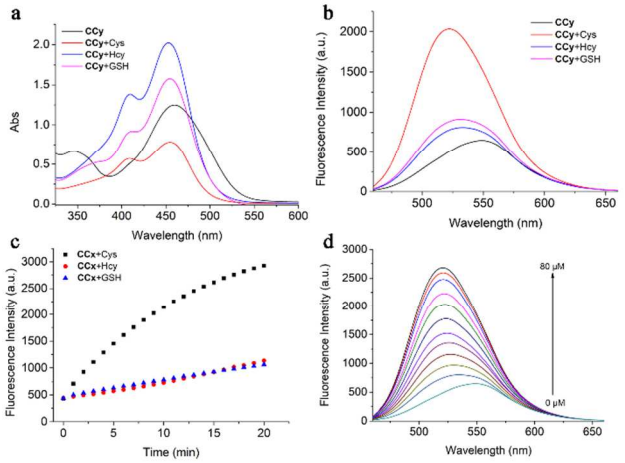


Figure 1. (a) UV/vis absorption spectra of **CCy** (2 μM) upon treatment with Cys, Hcy or GSH (40 μM). (b) Fluorescence spectra of **CCy** (2 μM) upon treated with Cys, Hcy or GSH (40 μM). (c) Time-dependent fluorescence intensity of **CCy** (2 μM) at 520 nm in the presence of Cys, Hcy or GSH (40 μM). (d) Fluores-

cence emission spectra of **CCy** (2 μM) in the presence of varied concentration of Cys (0~80 μM). Ex = 445 nm.

Selectivity of CCy. To evaluate the selectivity of **CCy**, we investigated the fluorescence spectra of **CCy** (2 μM) treated with Cys, Hcy, GSH or other 19 kinds of natural amino acids, common sulfureted anions and common metal ions(40 μM) in PBS buffer solution. These relevant species include Glutamic acid (Glu), Phenylalanine (Phe), Alanine (Ala), Proline (Pro), Threonine (Thr), Histidine(His), Isoleucine (Ile), Arginine (Arg), Lysine (Lys), Valine (Val), Leucine (Leu), Methionine (Met), Glutamine (Gln), Tryptophan (Trp), Serine (Ser), Glycine (Gly), Tyrosine (Tyr), Asparagine (Asn), Aspartic acid (Asp), $Mg(NO_3)_2$, $Al(NO_3)_3$, $Fe(NO_3)_3$, $Cu(NO_3)_2$, $ZnCl_2$, $Cd(NO_3)_2$, $Cr(NO_3)_3$, $CaCl_2$, Na_2S, Na_2SO_4, $Na_2S_2O_3$, $NaHSO_3$ and Na_2SO_3. **CCy** displayed remarkable fluorescence enhancement in the presence of Cys but inconspicuous increase when treated with Hcy or GSH (Fig. 2). There was no interference other than Cu^{2+} and Fe^{3+}. Cu^{2+} will coordinate with the di-ketone structure to quench the fluorescence. However, Cu^{2+} generally will not appear in body and there are almost no free Fe^{3+} in the organism, therefore will not interfere with the detection process. Under the same condition, the results showed that scarcely any influence on the detection of Cys was observed upon addition of other interfering species. All this clearly show that **CCy** has a selectivity towards Cys compared with other nucleophiles such as amino and hydroxyl. Moreover, digital photographs of **CCy** with different analytes under ambient light and 365 nm UV lamp were caught out (Fig. 2). Although no significant changes were observed with the naked eye, an obvious transform from weak yellow fluorescence to strong green fluorescence was obtained.

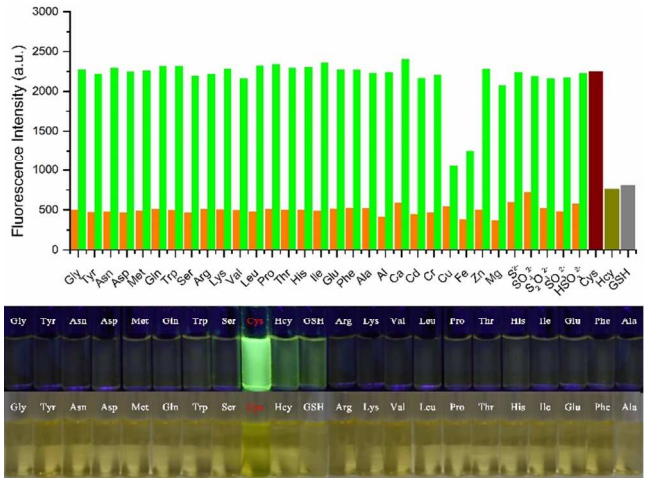


Figure 2. Up: selectivity and competitiveness of **CCy** (2 μM) to various analytes (40 μM) in PBS buffer. (The orange bars represent the fluorescence intensity of **CCy** after treated with analytes. Then the solution was treated with Cys, and the green bars show the fluorescence intensity of orange bars treated with Cys.) Down: color changes and fluorescence changes excited by UV lamp (365 nm) of **CCy** upon addition of Cys, Hcy, GSH and various amino acids.

Reaction mechanism and suitable pH. In order to confirm the sensing mechanism, Cys was added to d_6-DMSO solution of **CCy**, and the product was studied using HRMS. The partial ^1H NMR spectra of **CCy** and the product mixture were shown in Fig. S-17 and Fig. S-21. The resonance signal corresponding to the acryloyl proton at 6.24 ppm, 6.47 ppm and 6.59 ppm disappeared (Fig. S-24), consistent with previous reports. The crossover in Job's plot graph is 0.518, which show a 1: 1 stoichiometry between **CCy** and Cys (Fig. S-10).

Considering the further application in vivo, the influence of pH (from 1 to 13, prepared with hydrochloric acid and sodium hydroxide) on the fluorescence of **CCy** with Cys (40 μM) was taken into account. The emission intensity at 520 nm of **CCy** kept on a high level with pH value. It is observed that **CCy** can works well as a probe both in cytoplasm and lysosome.

Cytotoxicity and application in animal serum. As one of the important amino acids in cell, Cys can influence the activity of cells when its concentration changed. Therefore, before exploring the application for bio-imaging, the cell viability of **CCy** and compound **3** to HeLa (Fig. 3 and S-15) was measured using a standard MTT assay. When treating with 120 μM of **CCy** or compound **3**, the viability was more than 80% or 90% respectively, which demonstrates their low cytotoxicity and high feasibility in bio-experiment. Simultaneously, the capability of the probe to quantitatively detect Cys in animal serum (chicken, horse, sheep, cow and goat) was measured, and the Cys concentration of all the 5 kinds of serum were within normal range (Fig. 3a and S-11).

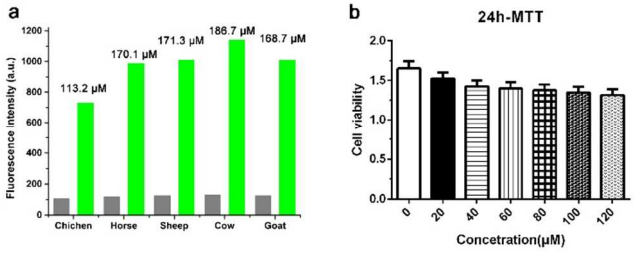


Figure 3. (a) Gray bar represent the value A (the fluorescence intensity of **CCy** with the thiol-blocking reagent N-ethylmaleimide in serum solution), the green bar represent the value B (the fluorescence intensity of **CCy** in serum solution), the number in the figure is the calculated Cys concentration. (b) MTT assay for the viability of HeLa cell treated with various concentrations (0-120 μM) of **CCy**. Error bars represent the standard deviations of 3 trials.

Bioimaging and the possibility to be biosensor. It is possibility to use **CCy** for fluorescence imaging in living cells and tissues because the insignificant cytotoxicity and suitable amphipathicity. This probe was applied for detecting endogenously generated Cys by incubating Hela cells. After incubated at 37 °C for 5–60 min with 10 μM **CCy**, it was observed that the green fluorescence gradually increased over time, and the strongest intracellular fluorescence was observed in 30 min (Fig. 4a). Moreover, when the absence of **CCy**, the unstimulated cells showed no intracellular fluorescence. All these suggested that endogenously generated Cys could easily be captured by **CCy**.

In most studies, fluorescence bioimaging of probe is successful, but few could quantitative detect intracellular Cys, thus the quantitative determination was simultaneously studied. It should be noticed that when more Cys was added with **CCy**, the fluorescence intensity enhanced (Fig. 4b). As expected, with increasing Cys concentration in the range of 0–400 μM, the brightness of cells show a linear increase.

Subsequently, HeLa cells were pretreated with N-ethylmaleimide (NEM, a thiol-blocking reagent) for 30 min to remove the endogenous cellular thiols and then 10 μM of **CCy** was added. After 10 min, there are no background fluorescence, which further confirmed that the fluorescence enhancement was caused by the reaction of **CCy** with cellular thiols (Fig. 4c). As another control experiment, the fluorescence intensity did not catch significant enhancement when cells were pretreated with 10mM of GSH or Hcy, which

demonstrated that **CCy** could detect Cys normally even in cells with high level of GSH or Hcy. In addition, when HeLa cells were pretreated with 1 mM Cys and further incubated with **CCy**, the observed fluorescence was much stronger. This experiment indicates that the reaction of **CCy** and Cys produced the green fluorescence.

To explore the capability of **CCy** to detect Cys in organisms, mice organ tissues was also investigated. After injected with the probe solution (100 μL, 1 mM/L; dimethyl sulfoxide: normal saline=1:1), the bright field and green channel photograph of various mice organ tissue slices was obtained (Fig. S-13). The results show that **CCy** can be distributed to the various organs (heart, liver, spleen, lung, kidney, intestine and testicle) of mice rapidly, which is conductive to the detection of Cys in different parts of mice. After that, the mice liver tissue slices were pre-treated with NEM, Cys (1 mM), Hcy (10 mM) or GSH (10 mM) before 50 μM of **CCy** added, and the similar phenomena with the cell imaging experiments was observed (Fig. S-12). The series of experiments showed **CCy** has great ability of imaging in tissue, and confirmed the selectivity of **CCy** to Cys in vivo.

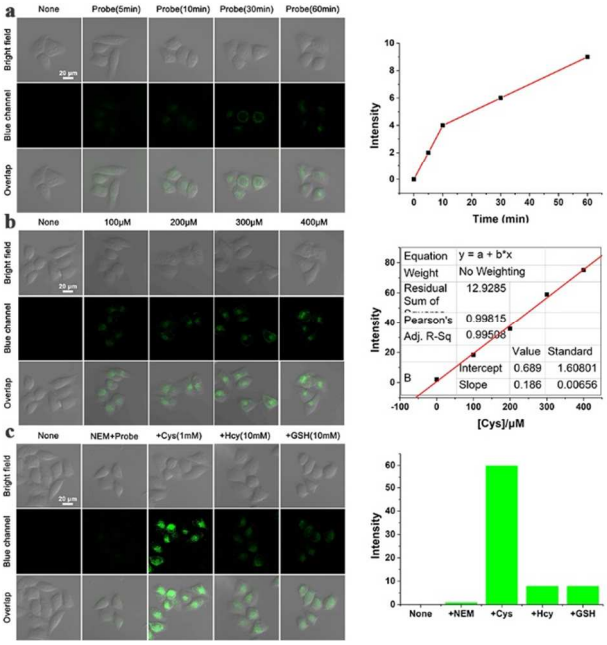


Figure 4. (a) Time-dependent images of HeLa cells treated with **CCy** and the scatter chart of intensity. (b) Images of HeLa cells treated with different concentration of Cys then added **CCy** for 10 min and the titration curve. (c) Images of HeLa cells treated with different methods and the bar chart of intensity. (Scale bar = 20 μM)

Colocalization experiment. The colocalization experiments determined the location of fluorescence emission by co-staining LysoTracker Red and **CCy**. After cells internalized with 10 μM **CCy** for 30 min, LysoTracker Red (100 nM) was added, then cells were continuously incubated for an additional 30 min. The green fluorescence image of **CCy** and red fluorescence image of LysoTracker Red was mainly overlapped, which indicated the ability of **CCy** to target lysosome (Fig. 5). The intensity profiles of the linear regions of interest (ROI) across cells stained with **CCy** and LysoTracker Red vary in close synchrony (Fig. 5l). The intensity of correlation plots (Fig. 4k) revealed a high Pearson's coefficient (0.894), and overlap coefficient was calculated to be 0.897. Overlap coefficients k_1 and k_2 were found to be 1.2 and 0.8 respectively. Meanwhile, the colocalization experiment was made by co-staining MitoTracker Red with **CCy**, and the results show very low overlapping ratio (Fig. S-14). As mitochondria-targeting probe, most of them has positive charge for the slightly basic environment of mitochondria. The diethylamino group of **CCy** just provide slightly acidic for lysosome. The above data demonstrated the specificity of **CCy** towards lysosome.

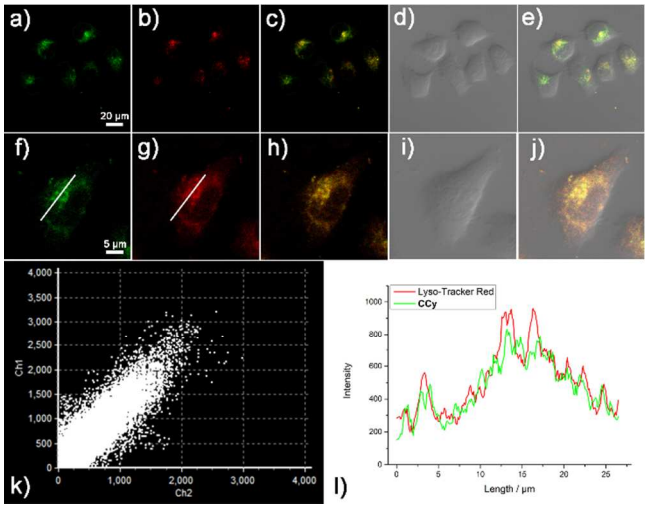


Figure 5. Fluorescence image of colocalization experiment in (a, f) green channel, (b, g) red channel and (c, h) overlap. And (d, i) is bright-field image, (e, j) is overlap. (k) The fluorescence intensity correlation plot. (l) Intensity profile of regions of interest (ROI) across HeLa cells.

Two-photon properties and the application in bioimaging. In the cases of simple cell imaging, one-photon (OP) microscopy is enough for image intracellular Cys. However, in a majority of these cases, the determined sample is tumor or other thick tissue, for which only two-photon (TP) microscopy can be used. **CCy** showed almost no fluorescence with very low quantum yield (0.025), while that of compound **3** is 0.060. Subsequently, for study the two-photon property of **CCy** and compound **3**, the TP integrated area and absorption cross section were measured[44]. At 820 nm, Compound **3** catches the δ_{max} value (557 GM), but the maximum integrated area appeared at 850 nm (Fig. S-16). Hence, in order to obtain the best effect in TP fluorescence experiment, 850 nm was selected as Ex. Meanwhile, the TP absorption cross section of **CCy** was much similar with that of compound **3** at 850 nm (80.6 GM for **CCy**, 83.3 GM for **3**).

Furthermore, in order to demonstrate the application of two-photon properties of **CCy**, fluorescent images of tumor tissues was achieved. In OP experiment, clear fluorescence imaging can only be caught before 100 μm, while for TP experiment it comes to 250 μm (Fig. 6). This experiment indicates that **CCy** has the potential to detect Cys in tissue using TP microscopy.

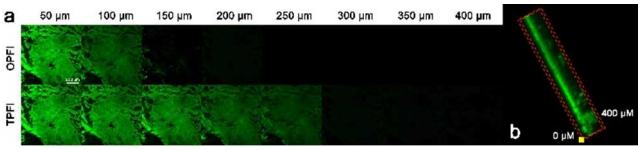


Figure 6. (a) The Z-scan TPE (TPFI) and OPE (OPFI) confocal fluorescence imaging of thick mice tumor tissue slice stained by **CCy** at di□erent penetration depths, the scale bar is 200 μm. (b) The 3D reconstruction from confocal Z-scan TPE imaging sections at a depth of 0-400 μm.

CONCLUSION

In summary, we prepared a coumarinocoumarin-based compound (**CCy**) and utilized it as a fluorescent biosensor for Cys in lysosome. **CCy** showed a rapid conjugate addition-cyclization reaction to afford a detection limit of Cys as low as the micromolar range in aqueous buffer. In addition, HeLa cells and mice tissues were successfully subjected to fluorescence imaging for detecting Cys in vivo. Then the cytotoxicity experiment of **CCy** achieved good results, and the two-photon fluorescent experiment demonstrated the advantages of the probe in detecting Cys within tissue. Meanwhile, the colocalization experiment proved the ability of **CCy** to target lysosome. The foregoing indicates that **CCy** is a biocompatible fluorescent probe that has the potential as a protein marker or disease surveillance tool. Because coumarinocoumarin has simple synthesis method and rich modification sites, our next plan is to modify its structure for better biocompatibility, selectivity and targeting. In the future, there will be more in-depth research for the fluorescence detection and imaging of biologically active molecules in vivo.

ASSOCIATED CONTENT

Supporting Information

Synthesis of compound **1** and **2**, structure characterizations of compound **3** and **CCy**, and additional data. The supporting Information is available free of charge on the ACS Publications website.

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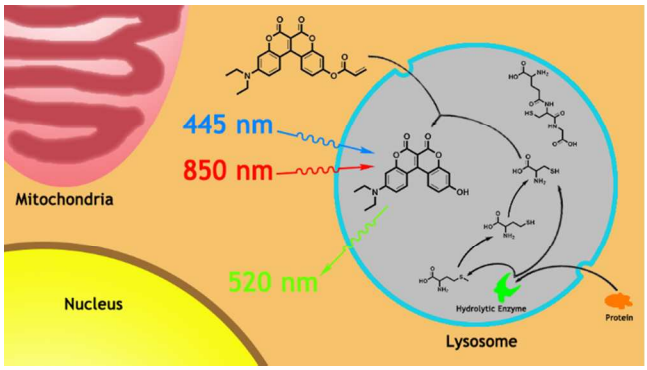
445 nm
850 nm
520 nm
Mitochondria
Nucleus
Hydrolytic Enzyme
Protein
Lysosome