Fluorescent Labeling of Dicysteine-Tagged Peptide for Monitoring and Optimization of Protein Bio-production in Bacteria

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Abstract

A rapid and convenient strategy to monitor the productivity of biomanufacturing is essential for the research in optimizing relevant bioprocesses. In this work, we have developed a fluorescein-derived probe (FL-DT) that reacts rapidly with thiol groups via 1, 4-Micheal addition reaction of the sulfhydryl to unsaturated ketone and releases fluorescence. FL-DT specifically forms fluorescent adduct with two adjacent thiols in a protein of interest (POI), making the probe a reliable tool for protein quantification. The production of xylanase fused with a short di-Cys tag was then successfully monitored and quantified with FL-DT in E. coli system under different protein expression conditions, providing useful information for optimizing the bioprocess. Our work provides a convenient and efficient strategy for POI labeling and monitoring bioproduction.

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Abstract: A rapid and convenient strategy to monitor the productivity of biomanufacturing is essential for the research in optimizing relevant bioprocesses. In this work, we have developed a fluorescein-derived probe (**FL-DT**) that reacts rapidly with thiol groups via 1, 4-Micheal addition reaction of the sulfhydryl to unsaturated ketone and releases fluorescence. **FL-DT**specifically forms fluorescent adduct with two adjacent thiols in a protein of interest (POI), making the probe a reliable tool for protein quantification. The production of xylanase fused with a short di-Cys tag was then successfully monitored and quantified with **FL-DT** in*E. coli* system under different protein expression conditions, providing useful information

for optimizing the bioprocess. Our work provides a convenient and efficient strategy for POI labeling and monitoring bioproduction.

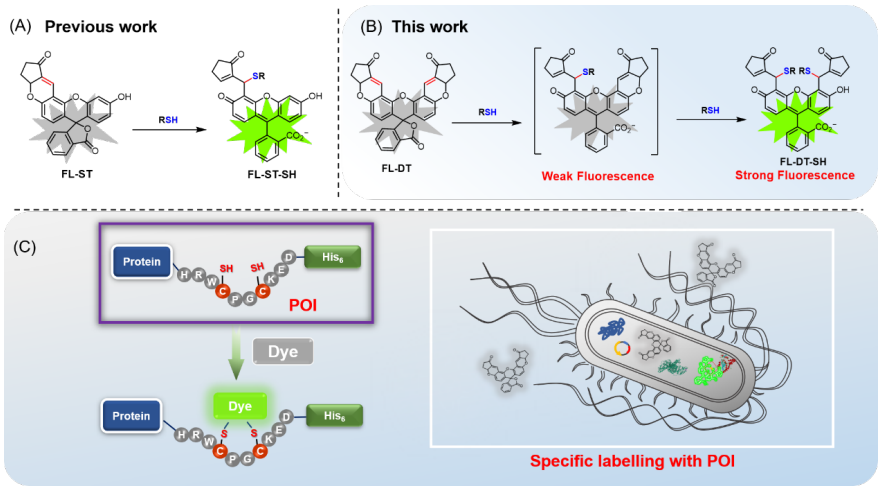
Keywords: dicysteine-tagged peptide, fluorescent probe, rapid labelling, expression conditions screening, xylanase

Introduction

Biomanufacturing of enzymes, proteins, therapeutics, and chemicals based on engineered cells or bacterium has been an emerging area that shows great importance. For instance, xylanase, which is mostly produced in the bacterial system, has enormous applications including waste-water treatment, pre-bleaching in the paper and pulp industry, bioconversion of biomass to biofuels, and other high-value final products, as well promoting the texture of nutrients in the food industry.[1, 2] The expression of biomanufacturing from laboratory to industry with reliable scale-up remains a tremendous economic and technical barrier, which requires substantial efforts in researching and optimizing these bioprocesses. The conventional protein quantification methods include Lowry method,[3] Bradford method,[4] Bicinchoninic acid (BCA) method,[5] etc.,[6] which are all time-consuming and economically inefficient due to involving cell breaking and protein purification processes. Thus, it is of great importance to develop convenient and rapid methods to monitor biomanufacturing productivity and this would facilitate the optimization of the bioprocesses.

Fluorescent labeling of a specific protein of interest (POI) with genetically encoded fluorescent proteins or fusing the POI to an enzyme tag are widely used approaches in chemical biology to study the expression, localization, and trafficking of the protein in live cells and organisms.[7, 8] However, the relatively large size of fused tags can sterically disturb the folding, functions, or even the localization of the POI.[9, 10] Alternatively, Tsien and coworkers developed small organic fluorophores, termed FlAsH and ReAsH, that are activated by binding to specific tetracystein motifs (e.g. CCPGCC).[11-13] This method allows the visualization of intracellular trafficking of recombinant proteins without the washing step.[14, 15] However, these arsenic probes have an ambiguously toxic impact on living cells,[16, 17] along with the uncertainty resulting from reversibility,[13, 16, 18] limiting their applications. Nevertheless, the small molecule protein-labeling strategy with a non-toxic fluorogenic agent would be promising to monitor enzymatic productivity.

Our previous work reported that the Michael addition reaction of thiol to α , β -unsaturated ketone in a 2-cyclopentenone modified fluorescein releases the etherified phenolic group (the ring-open of spiro group due to the nucleophilic attack of thiol), resulting in a fluorescence enhancement (Scheme 1A).[19] However, the monothiol responsive probe lacks specificity towards POIs.[20, 21] Herein, we report a new fluorescein-based protein-labeling agent (FL-DT), which is modified by two 2-cyclopentenone, making the probe emit fluorescent signals only when it reacts with two thiols simultaneously. This newly designed FL-DT reacts with a specific di-Cys containing peptide tag rapidly and shows promises in monitoring the production of POIs determining the total amount of protein expressed in *E. coli* system in a fast and highly specific way (Scheme 1B and 1C).



Scheme 1. The mechanism of (A) probe FL-ST with single reaction site in previous work and (B) probe FL-DT with two reaction sites in this work. (C) The design of fluorogenic peptide-tag labelling strategy with FL-DT.

Experimental section

Experiment and reagents

^{1}H NMR (Ascend 400, 400 MHz) and ^{13}C NMR (101 MHz) spectra were performed on Bruker spectroscopy. Mass spectra were measured on Q-Tof mass spectrometer (Agilent 6530). Fluorescence emission spectra were obtained on RF-5301/PC fluorophotometer (Shimadzu) and microplate detector (Bio Tek Instruments Synergy H1). The construction of recombinant protein was operated on Mastercycler persona PCR Amplifier (Eppendorf). Purified protein was obtained from AKTATM Prime protein purification system (GE) and Ni^{2+} affinity chromatography (GE). Enzyme analysis was carried out on Mini-Protein tetra cell Electrophoresis Apparatus (Tanon). The gel documentation was collected and analyzed by the Gel imager (Tanon-5200Multi). The cell images were observed by the inverted fluorescence microscope (Olympus IX73P1F). All reagents were used in the experiment directly are purchased on the market without further purification.

Synthesis

Synthesis of FL-DA

The fluorescent probe **FL-DA** required for these studies was synthesized as shown in **Scheme S1** . Fluorescein-dialdehyde (**FL-DA**) was obtained according to the previous work.[22, 23] Fluorescein (9.02 mmol, 3.00 g) and urotropine (45.13 mmol, 6.32 g) were dissolved in trifluoroacetic acid (25 mL). The resulting mixture was stirred vigorously and refluxed at 90 overnight. After the mixture was cooled to room temperature, 200 mL of dilute hydrochloric acid (4 mol/L) solution was added to get a turbid solution and stirred until clear. The solution was extracted with dichloromethane and saturated brine for several times, then the organic phase was dried with anhydrous sodium sulfate and collected by reduced pressure distillation. Chromatography on silica gel column using dichloromethane as the eluent afforded product as white solid.

Synthesis of FL-DT

FL-DT was synthesized based on the previous work of our group.**FL-DA** (1.00 mmol, 388 mg), 2-cyclopenten-1-one (4.00 mmol, 328 mg) and imidazole (2.00 mmol, 36 mg) were mixed in 15 mL of tetrahydrofuran and 10 mL of deionized water. The mixture was stirred at room temperature for two weeks. After the reaction finished, the crude solid was collected by reduced pressure distillation and further purified on a silica gel using dichloromethane/methanol (200:1, V / V) as the eluent to get a light-yellow solid

Synthesis of short peptides

All short peptides were ordered from a peptide company (DgPepetides co., ltd), which provided synthesis and purification. All sequences were confirmed by mass spectrometry report and high-performance liquid chromatograph (HPLC) analysis report to ensure that the purity was higher than 95%.

Fluorescence enhancement test

FL-DT was dissolved in methanol (MeOH) for stock solution (1 mM). The various analytes were prepared for aqueous solution (10 mM). The probe solutions were diluted with HEPES buffer (10 mM, pH 7.4, 1% CH_3CN) for testing fluorescent spectra. Except for Job's plot and detection of fusion protein, which are carried out on the Microplate Reader, the rest of the fluorescence signals are measured on the fluorophotometer.

SDS-PAGE gel electrophoresis and fluorescence imaging

The adducts of probe and protein were separated and analyzed by 12% SDS-PAGE. The proteins were labelled by probe at a ratio of 1:1 for 2 h. After mixing with 5× loading buffer and boiling for 5 minutes, the samples were added to each well with 20 μL. The voltage of electrophoresis was 120 V, and the gel was imaged by an imaging system on Gel imager (Tanon-5200Multi) directly (excited under blue light). As a control, the gel was stained by Coomassie brilliant blue and imaging under transmitted light at 302 nm.

Construction of expression vectors

The recombinant protein xylanase was amplified by polymerase chain reaction (PCR) using the genomic DNA extracted from *Bacillus subtilis* Lucky9 as template with primer pair F1 GGC*CATATG* ATGTTCAAATTCAAAAAAAA with restriction site NdeI and R1 GGC*CTCGAG* GTACCACACTGTTACGTTAG with restriction site XhoI. PCR amplification was carried out as follow: 95 °C for 5 min, 35 cycles including 95 °C for 30 s, 56 °C for 20 s, 72 °C for 90 s, and a final extension step at 72 °C for 10 min. The product of PCR was determined by 1% agarose gel electrophoresis and purified with PCR Clean up Kit to recovery. The amplified xylanase gene was inserted into the pET-28a expression vector after the digestion with NdeI and XhoI and confirmed by sequencing. Then the tag 10C2C was added at the C-terminal of xylanase sequence using the primer F2 GGC*CATATG* ATGTTCAAATTCAAAAAAAA (with cleavage sites of restriction enzyme NdeI) and R2 GGC*CTCGAG* GTCTTCTTTGCAACCCGGGCACCAACGGTGGTACCACACTGT (with cleavage sites of restriction enzyme XhoI) to amplify the sequence of xylanase-10C2C, and the PCR product was inserted into pET-28a expression vector after the digestion with NdeI and XhoI and confirmed by sequencing.

Expression and analysis of recombinant protein

The constructed pET-28a-xylanase-10C2C was transformed into *E. coli* BL21 (DE3) by heat shock (42 °C, 90 s). A single colony containing the recombinant plasmid was picked up from Luria broth (LB) agar plate. Luria broth media with kanamycin (50 mg/L) was inoculated with a glycerol stock of *E. coli* BL21 (DE3) containing constructed plasmids and grown overnight at 37 °C with shaking of 180 rpm. The overnight culture was conducted a 1/50 inoculation of 40 mL fresh Luria broth media containing 50 mg/L kanamycin, then was cultured until the cells density (OD_{600}) reached 0.4-0.6 (37 °C, 180 rpm). The induction conditions were optimized, including the inducer concentration, induction time and induction temperature. The recombined strain was grown for another 24 h, the cells were harvested by centrifugation for 10min (12,000 rpm, 4 °C), and the cell pellets were resuspended in 40 mL phosphate buffer (20 mM, pH 7.5), and lysed by ultrasonication (5 s, 3 s, total 10 min), then the supernatant of cell lysis (including cytoplasm and periplasmic fraction) was obtained by centrifugation for 10 min (12,000 rpm, 4 °C), the pellets by sonication were also resuspended in 40 mL phosphate buffer. The expression of proteins was analyzed by 12.5% SDS-PAGE.

Purification and quantitative detection of recombinant protein

After induction, the cell solution was centrifuged to remove the supernatant, adding 40 mL phosphate buffer (20 mM, pH 7.5) to resuspend the cell pellets for disruption. The crude enzyme was collected by crushing cell and centrifugation, and purified by affinity chromatography using Ni-NTA with His-tag in recombinant

protein. After recombinant protein N2C was eluted gradient with imidazole buffer (75-500 mM) and further purified by gel filtration (Superdex 200) with Tris-NaCl buffer (50 mM, pH 8.0), the concentrated protein was collected by ultrafiltration with a 10 kDa filter membrane. The purified recombinant protein was denatured in boiling water and determined by SDS-PAGE gel using 12.5% (*w/v*) separating gel and 4.0% (*w/v*) stacking gel for zymogram analysis. After electrophoresis, the gel was stained with Coomassie blue at 65 degC under shaking condition for 30 min. The quantitative detection of recombinant protein was determined by Bradford method (Coomassie brilliant Blue G-250 regent) with bovine serum protein as standard.

Living-cell imaging with specific labeling

After fermentation, the cell solution was centrifuged to remove the supernatant and resuspended with phosphate buffer (20 mM, pH 7.5). The cell pellets resuspension was co-incubated with **FL-DT** (10 μM) at 37 °C for 1 h. After removing the solution, the cell pellets were resuspended in deionized water and imaged under an inverted fluorescence microscope.

The microbial flow cytometry

The cell solution was obtained with post-processing as above description and treated with **FL-DT** (1:1) at 37 °C for 3 h. Before counting the single cells with fluorescence change on the flow cytometer via FITC channel, the samples were diluted to 20,000 cells per 10 μL. N2C and Xyn were expressed with 0.5 mM IPTG at 30 °C for 20 h. NI is the N2C which was expressed without IPTG expression at 30 °C for 20 h.

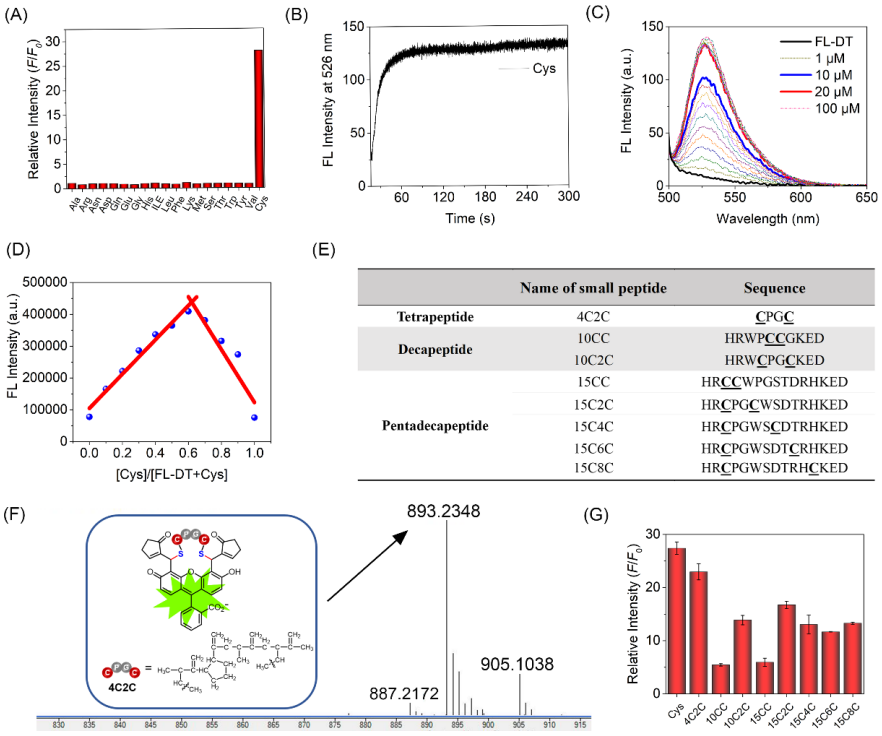
Results and discussion

3.1 Fluorescent properties of probe to small molecular thiols

To verify the thiol-specific reactivity of **FL-DT** , the probe was first mixed with 10 equivalents of various amino acids in HEPES buffer, respectively. As shown in **Figure 1A** , only the thiol-containing Cys triggers the fluorescence enhancement ($?_{max} = 526$ nm), which is ˜ 28-fold higher than the cases of other analytes. The results demonstrate that **FL-DT** specifically responds to the thiol group (**Figure S1A**). We next performed the kinetics studies for the fluorescent responses of **FL-DT** to Cys at 526 nm. As shown in **Figure 1B** , upon the addition of Cys, the reaction between α , β -unsaturated ketones, and thiols complete within 30 sec as the fluorescence intensity reaches a plateau. Additionally, the rate constants of the consecutive thiol addition were measured by the resulting time-dependent fluorescence intensity with a large excess Cys (**Figure S4** and **Table S1**). The first thiol addition is 25 times faster than the second addition. The probe**FL-DT** (10 μM) was then titrated by Cys (0-100 μM) to lead the fluorescence intensity at 526 nm emitted from the activated**FL-DT** enhanced gradually and determine its limit of detection (LOD) as 0.32 μM for thiol group (**Figure 1C** and **S1B**). The mechanism of the fluorescent response of **FL-DT** was further investigated to support our assumption (**Scheme 1**). Mass spectrometry analysis of the product obtained from **FL-DT**reacted with Cys in HEPES buffer exhibits a peak at 757.25, which corresponds to [**FL-DT** -2Cys-H]$^-$(**Figure S1C**). The stoichiometry between **FL-DT** and Cys was also determined to be 1:2 from a Job's plot (**Figure 1D**). The obtained binding stoichiometry is consistent with the mass spectrometry analysis, demonstrating that one **FL-DT** molecule eventually reacts with two thiols. This event is required to trigger the activation of fluorescence enhancement of fluorescein since the presence of etherified phenol group by 2-cyclopentenone quenches the emission.[19]

In order to label a protein with **FL-DT** , fusing a diCys-containing peptide sequence to POI is necessary. As reported previously, the properties of the peptide sequence, including the conformation and the distance between Cys residues, affect the performance of the labeling agent significantly.[13, 24]In this work, we chose a hairpin sequence (-CPGC-) as the template to design the diCys-containing peptides because hairpin is reported to be a preferred conformation for multiple-thiol binding.[13] A number of peptides with varying lengths and distances between two Cys residues (**Figure 1E**) were prepared and tested for inducing the fluorescence response of **FL-DT** . According to the titration experiments, the fluorescence enhancement from the thiol reacted**FL-DT** generally reached a maximum when 1 equivalent of each peptide was added (**Figure S2**), while the reaction between**FL-DT** and each peptide mostly completed within 60 s (**Figure**

S3). Moreover, the shortest peptide 4C2C induces the strongest fluorescence enhancement, which is ˜ 80% of that induced by Cys (**Figure 1G**). Increasing the length of the peptide tag to 10 and 15 amino acids (10C2C and 15C2C) generally reduces the fluorescence intensity to ˜ 60% of that induced by Cys, and the role of the distance between two Cys residues (15C2C, 15C4C, 15C6C, and 15C8C) is not critical; however, the direct connection of Cys residues (10CC and 15CC) causes a low fluorescence efficiency (**Figure 1G**), demonstrating the necessity of a spacer between the two Cys. Mass spectrometry was also carried out on the product from the reaction between **FL-DT** and the tetrapeptide 4C2C in HEPES buffer. The peak at 893.2348 represents for [**FL-DT** -4C2C-H]$^-$ (**Figure 1F**), confirming that **FL-DT** preferably reacts with two thiols on one peptide chain, thus, reliable for quantifying the diCys-containing peptide.



	Name of small peptide	Sequence
Tetrapeptide	4C2C	CPGC
Decapeptide	10CC	HRWPCCGKED
	10C2C	HRWCPGCKED
Pentadecapeptide	15CC	HRCCWPGSTDRHKED
	15C2C	HRCPGCWSDTRHKED
	15C4C	HRCPGWSCDTRHKED
	15C6C	HRCPGWSDTCRHKED
	15C8C	HRCPGWSDTRHCKED

Figure 1 . (A) Fluorescence enhancement multiple of responses of **FL-DT**to various amino acids. (B) Time-dependent fluorescence intensity of**FL-DT** (10 μM) at 526 nm in the presence of 100 μM Cys. (C) Fluorescent titrations of **FL-DT** in response to the addition of Cys (0-100 μM). (D) Job's plot of the reaction between **FL-DT**and Cys in HEPES buffer. (E) Specific names of a series of small peptides. (F) The ESI-MS spectrum of **FL-DT** reacted with 4C2C in HEPES buffer. (G) Relative fluorescence responses of **FL-DT** (10 μM) to 4C2C, 10CC, 10C2C, 15CC, 15C2C, 15C4C, 15C6C and 15C8C (100 μM) in HEPES buffer. λ_{ex} = 490 nm, Slit: 5 nm/3 nm.

3.2 Thiols-containing proteins specific labelling

Next, to demonstrate that **FL-DT** can be linked to thiols-bearing proteins and release fluorescence, bovine serum albumin (BSA), containing 17 pairs of disulfide bonds and one Cys existed as a free sulfhydryl group, [23, 25, 26] was selected as a model protein for the test and probe **FL-ST** with one single reaction site was used for comparison. As shown in **Figure 2A** ,**FL-ST** releases fluorescence upon the addition of BSA by reacting with the single free Cys, while no change in fluorescence is observed in the case of **FL-DT** . After the disulfide bonds in BSA were reduced with tris(2-carboxyethyl) phosphine (TCEP), the reduced form of BSA (rBSA) was fluorescently labeled with **FL-DT**successfully (**Figure 2A**). The results suggested that, due to the steric effect of BSA, **FL-DT** could hardly connect the monoCys between two BSA molecules simultaneously; while

the released thiol groups from the disulfide bonds would allow the BSA-labeling of**FL-DT** . The fluorescent rBSA-**FL-DT** adducts were then separated and analyzed by sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). An obvious fluorescent band corresponding to 66.4 kDa was observed for **FL-DT** treated rBSA, confirming the fluorescently labeled rBSA with **FL-DT** (**Figure 2B**). Taken together,**FL-DT** is a suitable labeling agent for protein bearing abundant and spatially adjacent Cys residues.

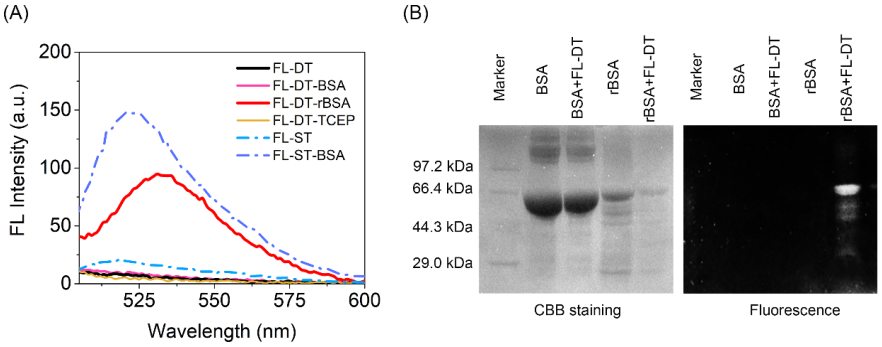


Figure 2. (A) Fluorescence responses of **FL-ST** (10 μM) to BSA (5 mg/mL) and **FL-DT** (10 μM) to solutions of BSA (5 mg/mL), rBSA and the sulfhydryl reducing agent TCEP (12.5 mM). λ_{ex} = 490 nm, Slit: 5 nm/3 nm. (B) SDS-PAGE analysis of the specific labeling of dithiol groups in proteins by **FL-DT** .

3.3 Specific recognition of fusion protein

We then moved forward to the application of monitoring the expression of POIs in live cells by employing **FL-DT** as the protein-labeling agent. Xylanase- expressing *E. coli* was selected as the model system for this investigation. Since xylanase has no Cys residue, a diCys-containing peptide sequence was fused to the C-terminus of xylanase through protein engineering. Considering the potential steric effect, the easiness, and the cost for protein expression, peptide 10C2C was selected as a tag motif. The recombinant xylanase (N2C) was expressed and purified as described in experimental section. The successful expression of N2C and specific labeling with **FL-DT**was confirmed by zymogram analysis with its corresponding band at approximately 25 kDa with distinct fluorescence observed, while the original xylanase (Xyn, about 22 kDa) had no fluorescence response to**FL-DT** (**Figure 3A**and **Figure 3B**). Also, no crosslinking was observed (**Figure S5**), demonstrating one **FL-DT** molecule only bind to one protein. To verify that the enhancement of fluorescence intensity was ascribed to the introduction of di-Cys rather than other amino acids, both Cys residues in the tag were mutated to alanine by constructing a new fused xylanase (NOC) (sequence described in supplementary materials). The results showed that mixing NOC with**FL-DT** barely generates fluorescence (**Figure 3B**). Furthermore, when the thiol groups in N2C pre-reacted with N-methylmaleimide (NMM), the protein lost the ability to activate**FL-DT** as well (**Figure 3B**). These results demonstrated that the **FL-DT** can respond to the recombinant xylanase that contains a CPGC motif with high selectivity and sensitivity. Additionally, the enzyme activity assays of the purified recombinant proteins were measured by dinitrosalicylic acid reagent (DNS) method based on a standard curve of the Xylose versus optical density ($OD_{540\ nm}$) (**Figure 3C**). The results show that the activity of N2C was almost the same as that of the original enzyme, while the N2C enzyme activity after being labeled with **FL-DT**slightly decreased (**Figure 3D**), suggesting that the**FL-DT** -based protein labeling approach could be a convenient tool for monitoring the production of POIs without impairing the protein function.

To further demonstrate the potential of **FL-DT** in monitoring the bioproduction of POIs, we then applied **FL-DT** in a N2C-expressing *E. coli* system. The **FL-DT** treated N2C-expressing *E. coli* cells exhibited enhancing fluorescence within 30 min (**Figure S7A**), demonstrating that **FL-DT**molecules are able to penetrate the cell membranes and successfully label the expressed N2C *in-situ* . After staining with**FL-DT** (10 μM) for one hour under 37 °C, apparent green fluorescence was observed within N2C-expressing *E. coli* cells under an inverted fluorescence microscope, while the cells containing Xyn showed almost non-

fluorescence change (**Figure 4A**). In addition, the **FL-DT** stained *E. coli* cells were analyzed via flow cytometry. The *E. coli* cells with a different abundance of expressed N2C (**Figure S6**) were selectively distinguished (**Figure 4B**). These results support that **FL-DT** enables the specific labeling and imaging of a POI in live cells, as well as the screening of cells with high productivity.

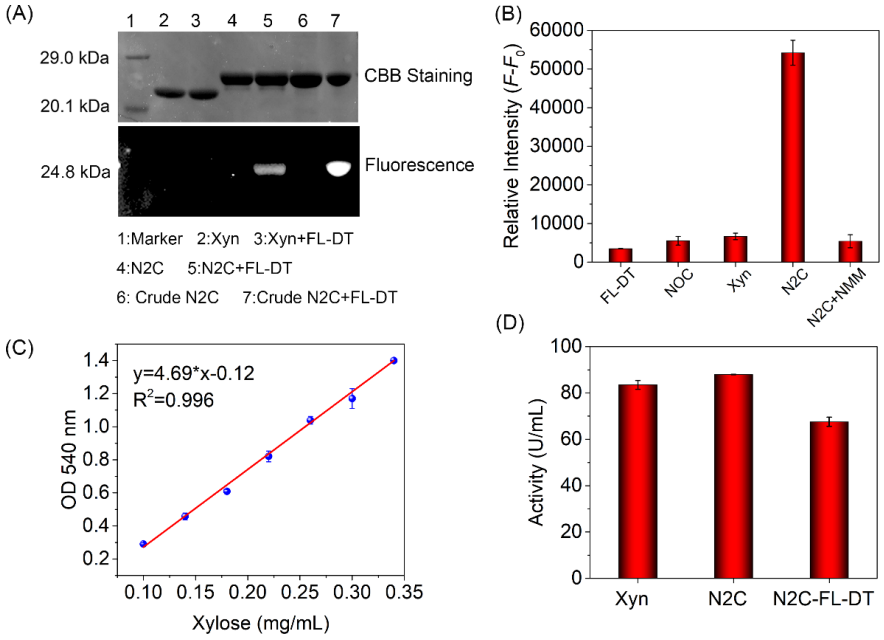


Figure 3. (A) SDS-PAGE experiments of labeling protein Xyn (original xylanase), N2C (fused protein with peptide tag after purification) and Crude N2C (fused protein with peptide tag) with **FL-DT** . (B) Relative fluorescence intensity of responses of **FL-DT** (1 μM) to various recombinant protein NOC, Xyn, N2C and N2C (0.1 mg/mL) after reaction with sulfhydryl masking agent NMM (100 μM), (n=3, error bars represent SD). (C) Standard curve of Xylose concentration (0.1-0.34 mg/mL) and OD (optical density) at 540 nm, (n=3, error bars represent SD). (D) Enzyme activity assay of pure protein Xyn, N2C, and N2C-FL-DT adducts, the concentration of proteins is 0.5 mg/mL, (n=3, error bars represent SD).

3.4 Optimization of production conditions for POI

A convenient quantification method for the expressed POI is essential for the optimization of bioproduction conditions. The relationship between **FL-DT** fluorescence intensity and the concentration of N2C was then investigated by incubating 0.01-1.00 equivalent of N2C with**FL-DT** for 30 min (**Figure S7B**). The presence of N2C triggered the release of fluorescence, which is also observed under a handheld UV lamp (**Figure 4C**). The changes in fluorescence intensity were in a linear relationship with the concentrations of the added N2C (**Figure S7C**). The limit of detection was determined to be 2 nM. The concentrations of N2C expressed in *E. coli* under variable production conditions, including the concentration of inducer isopropyl-β -D-thiogalactopyranoside (IPTG), induction time, and incubation temperature, were then quantified with **FL-DT** to determine the optimal parameters. Initially, three IPTG concentrations (0.1 mM, 0.5 mM, and 1.0 mM) were investigated for their impacts on protein expression. Each test group was subjected to 3 parallel experiments at 30 °C with an incubation time of 20 h. The fluorescence intensity was recorded and converted to protein concentration (method described in supplementary materials). The result reveals that the concentration of inducer IPTG barely affects the N2C production efficiency (**Figure S7D**). By fixing the inducer concertation at 0.5 mM, the effect of different induction temperatures (20 °C, 25°C, 30°C, 37°C) on protein expression at 20 h was explored, showing an optimal induction temperature of 30°C (**Figure 4D**). The contents of N2C expression after 8 h, 12 h, 16 h, 20 h, and 24 h were then compared under 30°C

with the inducer concertation of 0.5 mM(**Figure 4E**). The effect of induction temperature and culture time have a remarkable influence on the expression of recombinant protein, and the optimized condition for N2C protein production of ˜0.48 mg/mL is to incubate for 20 h at 30 °C.

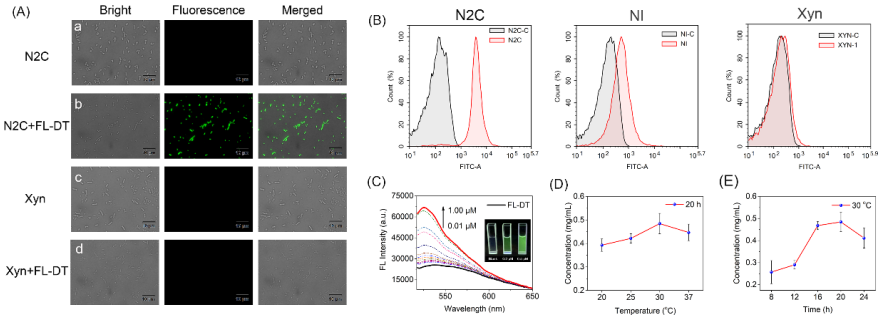


Figure 4 . (A) Specific labeling of N2C-expressing cells with **FL-DT** (10 μM). The N2C-expressing cells were treated without (a) and with (b)**FL-DT** , the Xyn-expressing cells were treated without (c) and with (d) **FL-DT** . Scale bar: 10 μm. (B) Flow Cytometry data of N2C (containing 0.5 mM IPTG as inducer), NI (no inducer), Xyn negative control samples (black line) and the samples treated with **FL-DT**(red line). (C) Fluorescence spectra of **FL-DT** (1 μM) with addition of different equivalents of N2C (0.01-1.00 μM). Photograph of**FL-DT** reacted with N2C (0 μM, 0.2 μM and 0.5 μM) visualized under a handheld UV lamp (inserted figure). Expression concentrations of recombinant protein with 0.5 mM of IPTG (D) at different temperature for 20 h, and (E) at different time periods at 30 °C.

Conclusion

In summary, we have developed a new fluorescein-derived protein-labeling agent FL-DT for monitoring the production of POIs in bacterial systems. The probe releases fluorescence when its α , β -unsaturated ketone moieties react with two thiols. Further characterizations prove that FL-DT specifically forms a fluorescent adduct with one peptide tag containing two adjacent thiol groups within 1 min, offering reliability in protein quantification. The application of monitoring protein production is successfully performed using fluorescence microscopy and flow cytometry in *E. coli* system that expresses xylanase fused with a CPGC-containing tag. The quantification of the POI can be achieved at a high specificity without breaking the cells. Thus, this new labeling agent provides a convenient tool for rapid POIs labeling and its applications include but are not limited to monitoring protein production and optimization of protein expression conditions.

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Conflict of interest

The authors declare no competing interest.

Data availability statement

Additional data related to this article are available in supplementary material.

Reference

1. Yu, J.,Liu, X.,Guan, L., et al. High-level expression and enzymatic properties of a novel thermostable xylanase with high arabinoxylan degradation ability from Chaetomium sp. suitable for beer mashing.*Int. J. Biol. Macromol.* **2021,** *168* , 223-232, doi:10.1016/j.ijbiomac.2020.12.040

2. Chang, S.,Guo, Y.,Wu, B., et al. Extracellular expression of alkali tolerant xylanase from Bacillus subtilis Lucky9 in E. coli and application for xylooligosaccharides production from agro-industrial waste. *Int. J. Biol. Macromol.* **2017,** *96* , 249-256, doi:10.1016/j.ijbiomac.2016.11.032

3. Li, J.,Zhao, Y. and Jiang, X. Quantitative analysis of protein in thermosensitive hydroxypropyl chitin for biomedical applications.*Anal. Biochem.* **2020,** *599* , 113745, doi:10.1016/j.ab.2020.113745

4. Compton, S. J. and Jones, C. J. Mechanism of dye response and interference in the Bradford protein assay. *Anal Biochem***1985,** *151* , 369-374, doi:10.1016/0003-2697(85)90190-3

5. Smith, P. K.,Krohn, R. I.,Hermanson, G. T., et al. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985,***150* , 76-85, doi:10.1016/0003-2697(85)90442-7

6. Maehre, H. K.,Dalheim, L.,Edvinsen, G. K., et al. Protein determination-method matters. *Foods* **2018,** *7* (1), 5, doi:10.3390/foods7010005

7. Chen, Y.,Tsao, K.,Acton, S. L., et al. A green BODIPY-based, super-fluorogenic, protein-specific labelling agent. *Angew. Chem. Int. Ed. Engl.* **2018,** *57* (38), 12390-12394, doi:10.1002/anie.201805482

8. Thiel, Z.,Nguyen, J. and Rivera-Fuentes, P. Genetically encoded activators of small molecules for imaging and drug delivery.*Angew. Chem. Int. Ed. Engl.* **2020,** *59* (20), 7669-7677, doi:10.1002/anie.201915521

9. Hatai, J.,Prasad, P. K.,Lahav-Mankovski, N., et al. Assessing changes in the expression levels of cell surface proteins with a turn-on fluorescent molecular probe. *Chem. Commun. (Camb.)* **2021,***57* (15), 1875-1878, doi:10.1039/d0cc07095e

10. Roßmann, K.,Akkaya, K. C.,Poc, P., et al. N-Methyl deuterated rhodamines for protein labelling in sensitive microscopy. *Chem. Sci.* **2022** , doi:10.1039/d1sc06466e

11. Griffin, B. A.,Adams, S. R. and Tsien, R. Y. Specific covalent labeling of recombinant protein molecules inside live cells.*Science* **1998,** *281* (5374), 269-272, doi:10.1126/science.281.5374.269

12. Martin, B. R.,Giepmans, B. N.,Adams, S. R., et al. Mammalian cell-based optimization of the biarsenical-binding tetracysteine motif for improved fluorescence and affinity. *Nat. Biotechnol.***2005,** *23* (10), 1308-1314, doi:10.1038/nbt1136

13. Adams, S. R.,Campbell, R. E.,Gross, L. A., et al. New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: synthesis and biological applications. *J. AM. CHEM. SOC.***2002,** *124* , 6063-6076, doi:10.1021/ja017687n

14. Gaietta, G.,Deerinck, T. J.,Adams, S. R., et al. Multicolor and electron microscopic imaging of connexin trafficking. *Science***2002,** *296* (5567), 503-507, doi:10.1126/science.1068793

15. Zhang, J.,Campbell, R. E.,Ting, A. Y., et al. Creating new fluorescent probes for cell biology. *Nat. Rev. Mol. Cell Biol.***2002,** *3* (12), 906-918, doi:10.1038/nrm976

16. Langhorst, M. F.,Genisyuerek, S. and Stuermer, C. A. Accumulation of FlAsH/Lumio Green in active mitochondria can be reversed by beta-mercaptoethanol for specific staining of tetracysteine-tagged proteins. *Histochem. Cell Biol.* **2006,** *125* (6), 743-747, doi:10.1007/s00418-005-0136-3

17. Strmiskova, M.,Tsao, K. and Keillor, J. W. Rational design of a highly reactive dicysteine peptide tag for fluorogenic protein labelling. *Org. Biomol. Chem.* **2018,** *16* (34), 6332-6340, doi:10.1039/c8ob01417e

18. Chen, Y.,Clouthier, C. M.,Tsao, K., et al. Coumarin-based fluorogenic probes for no-wash protein labeling. *Angew. Chem. Int. Ed. Engl.* **2014,** *53* (50), 13785-13788, doi:10.1002/anie.201408015

19. Chen, X.,Ko, S. K.,Kim, M. J., et al. A thiol-specific fluorescent probe and its application for bioimaging. *Chem. Commun. (Camb.)***2010,** *46* (16), 2751-2753, doi:10.1039/b925453f

20. Ma, K.,Zhao, L.,Yue, Y., et al. Thiol "Click" Chromene Ring Opening and Subsequent Cascade Nucleophilic Cyclization NIR Fluorescence Imaging Reveal High Levels of Thiol in Drug-Resistant Cells. *Anal. Chem.***2020,** *92* (24), 15936-15942, doi:10.1021/acs.analchem.0c03362

21. Huo, F. J.,Sun, Y. Q.,Su, J., et al. Chromene "lock", thiol "key", and Mercury(ii) ion "hand": a single molecular machine recognition system. *Org. Lett.* **2010,** *12* , 4756-4759, doi:10.1021/ol101771j

22. Xiong, X.,Song, F.,Sun, S., et al. Red-Emissive Fluorescein Derivatives and Detection of Bovine Serum Albumin. *Asian J. Org. Chem.* **2013,** *2* (2), 145-149, doi:10.1002/ajoc.201200109

23. Zheng, S.,Peng, J.,Jiang, L., et al. A rhodol-derived probe for intracellular biothiols imaging and rapid labelling of sulfhydryl-containing proteins. *Sens. Actuators, B.***2022,** *367* , 132148, doi:10.1016/j.snb.2022.132148

24. Guy, J.,Castonguay, R.,Campos-Reales Pineda, N. B., et al. De novo helical peptides as target sequences for a specific, fluorogenic protein labelling strategy. *Mol. Biosyst.* **2010,** *6* (6), 976-987, doi:10.1039/b918205e

25. Silva, M.,Faustino, H.,Coelho, J. A. S., et al. Efficient amino-sulfhydryl stapling on peptides and proteins using bifunctional NHS-activated acrylamides. *Angew. Chem. Int. Ed. Engl.***2021,** *60* (19), 10850-10857, doi:10.1002/anie.202016936

26. Pan, X.,Liang, Z.,Li, J., et al. Active-site-matched fluorescent probes for rapid and direct detection of vicinal-sulfydryl-containing peptides/proteins in living cells. *Chem. Eur. J.* **2015,***21* (5), 2117-2122, doi:10.1002/chem.201405349

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