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Communication

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Proximity-Induced Covalent Labeling of Proteins with a Reactive Fluorophore-Binding Peptide Tag

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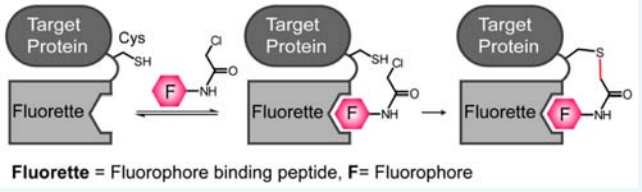
Supporting Information

ABSTRACT: Labeling of proteins with fluorescent dyes in live cells enables the investigation of their roles in biological systems by fluorescence microscopy. Because the labeling procedure should not disturb the native function of the protein of interest, it is of high importance to find the optimum labeling method for the problem to be studied. Here, we developed a rapid one-step method to covalently and site-specifically label proteins with a TexasRed fluorophore in vitro and in live bacteria. To this end, a genetically encodable TexasRed fluorophore-binding peptide (TR512) was converted into a reactive tag (ReacTR) by adjoining a cysteine residue which rapidly reacts with N-α-chloroacetamide-conjugated TexasRed fluorophore owing to the proximity effect; ReacTR tag first binds to the TexasRed fluorophore and this interaction brings the nucleophilic cysteine and the electrophilic N-α-chloroacetamide groups in close proximity. Our method has several advantages over existing methods: (i) it utilizes a peptide tag much smaller than fluorescent proteins, the SNAP, CLIP, or HaLo tags; (ii) it allows for labeling of proteins with a small, photostable, red-emitting TexasRed fluorophore; (iii) the probe used is very easy to synthesize; (iv) no enzyme is required to transfer the fluorophore to the peptide tag; and (v) labeling yields a stable covalent product in a very fast reaction.



Fluorette = Fluorophore binding peptide, **F**= Fluorophore

Labeling proteins with fluorescent moieties in living systems allows for the elucidation of their functions in biological contexts by fluorescence microscopy. Green fluorescent protein (GFP) and its variants,[1,2] SNAP- and CLIP-tag,[3,4] HaloTag,[5] and DHFR[6] are among the most common methods to label proteins in living systems. While these methods have been successfully applied to image proteins in live cells, the large size of the tags may alter the native functions of the protein of interest. To circumvent this problem, small peptide tags have been developed, such as the Q-tag,[7] ybbr,[8] A1/S6,[9] aldehyde tag,[10,11] AP-tag,[12] and LAP-tag,[13] which can be site-specifically and covalently labeled with small molecules by various post-translational modification enzymes.[14] On the other hand, nonenzymatic methods for site-specific covalent labeling of small peptide tags have been less studied despite their simplicity. The best-known example of this class is the FlAsH/ReAsH technology, which uses a tetracysteine recognition motif,[15−17] while other members include the tetraserine tag reacting with bisboronic acid probes,[18] a hydrazide-reactive peptide tag (HyRe),[19] the reactive tetraaspartate tag,[20] and a reactive His-tag.[21,22]

Fluorophore-binding aptamers as tools to image RNA molecules have increasingly gained in importance and attracted much scientific interest over the years.[23−29] However, fluorophore-binding peptides, dubbed "fluorettes", have not been widely utilized for protein labeling, albeit there is great potential. Fluorettes have been first developed for protein imaging and detection purposes by Rozinov et al. using phage display technology.[30] It was later shown that TexasRed-binding peptide (TR512) binds to the TexasRed fluorophore non-covalently with high affinity ($K_{avidity}$ of 25 pM on the phage surface), and TR512 has been successfully employed in live cells for imaging calcium dynamics on the cell surface.[31] Nevertheless, covalent labeling of proteins can be more desirable in specific applications where prolonged imaging periods and high signal-to-noise ratios are required. Toward this end, we describe here the conversion of a noncovalent fluorette system into a covalent protein labeling approach by proximity-induced reactivity. The concept is shown in Figure 1: To label proteins covalently and site-specifically with the TexasRed fluorophore, we added two cysteine residues as nucleophiles to the N-terminus of TR512 peptide, creating a Reactive TexasRed binding (ReacTR) tag, and an N-α-chloroacetamide group as a mild electrophile to the TexasRed fluorophore (Figure 1a and b). If the high-affinity binding of Texas Red fluorophore to ReacTR tag brings the electrophile into close proximity of the cysteine nucleophile, a stable covalent thioether bond should be formed between the fluorophore and the peptide tag. As the reaction rate is expected to increase with the local concentration of the nucleophile, we introduced two cysteine residues instead of one to the N-terminus of TR512 peptide.

First, we tested our design in vitro on recombinant maltose binding protein (MBP) fused to the ReacTR tag at the C terminus (MBP-ReacTR). To generate the mildly electrophilic

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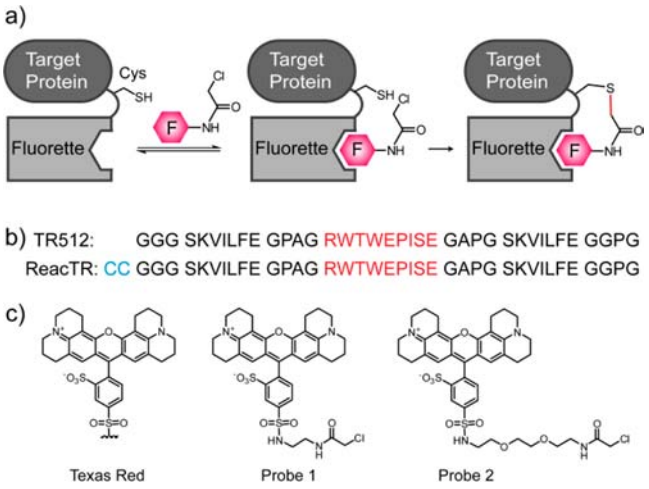

Figure 1. (a) Schematic representation of the covalent labeling approach of target proteins with a reactive fluorette (fluorophore binding peptide). The fluorette and the target protein are fused together and a cysteine residue was placed between them. Upon addition of the fluorophore conjugated to an N-α-chloroacetamide functional group, the fluorophore will first bind to the fluorette and then the nearby Cys reacts with the N-α-chloroacetamide, forming a covalent bond between the fluorophore and the fluorette. F denotes a fluorophore. (b) Sequences of the TR512 and reactive TR512 peptide tag (ReacTR). Blue: Reactive cysteine amino acids, Red: TexasRed fluorophore binding domain. (c) Chemical structures of the TexasRed fluorophore, Probe 1 and Probe 2.

fluorophore, TexasRed was conjugated to N-α-chloroacetamide via a short ethylene diamine linker (Figure 1c, probe 1). After incubating probe 1 (20 μM) with MBP-ReacTR (5 μM) at 37 °C for 30 min, samples were loaded onto an SDS-PAGE gel, and analyzed by fluorescence scanning as well as Coomassie staining. A strong fluorescent band corresponding to the size of MBP-ReacTR (~47 kDa) was observed in the gel, indicating the formation of a covalent bond between the fluorophore and protein (Figure 2a). On the other hand, no fluorescence was detected in the control experiments with MBP or MBP-TR (without the reactive cysteine residue), showing the specificity of the labeling reaction and necessity of cysteine residues for the formation of the covalent bond between the fluorophore and protein (Figure 2a). In addition, the presence of TexasRed fluorophore (free acid) efficiently inhibited the labeling reaction of MBP-ReacTR with probe 1, suggesting a specific interaction between ReacTR tag and probe 1 (Figure S1).

We then studied the kinetics of the reaction in detail. Samples were taken from the labeling reaction of MBP-ReacTR with probe 1 at different time points and in-gel fluorescence analysis was carried out (Figure 2b). The fluorescence intensity of the protein band was found to increase over time, and it was quantified by using a TexasRed conjugated reference protein where the amount of modification was quantified by UV−vis spectroscopy. About 50% labeling efficiency was observed after 20 min (Figure 2c), and after 100 min, the labeling yield was determined to be 84%. The second-order rate constant was calculated as 1100 M^{-1} s^{-1} (Figure S2), which is comparable to previously reported protein labeling reactions based on a reactive His-tag,[21] CLIP-tag,[4] or on a tetrazine ligation,[32] a fast bio-orthogonal click reaction. However, it is about ~25- and ~65-fold slower than SNAP-tag[4] and FlAsH-tag[17] based labeling reactions, respectively.

To investigate the influence of proximity on the kinetics of the labeling reaction, we changed the linker length in the probe as well as in the fusion protein between the tag and the target protein. Probe 2 with a longer linker between fluorophore and N-α-chloroacetamide was synthesized (Figure 1c), and the positions of the cysteine residues were shifted further away from the TR512 peptide tag by inserting two glycine residues, yielding MBP-CC-GG-TR protein. The highest labeling efficiency was observed when the linker length in both probe and peptide tag was minimal, presumably owing to higher local concentration of the reactive groups with respect to each other (Figure 2d).

As the next step, we examined the labeling of proteins directly in live *E. coli*. Bacteria harboring the plasmids for MBP, MBP-TR, or MBP-ReacTR were grown in LB medium, and the protein expression was induced by addition of IPTG (1 mM).

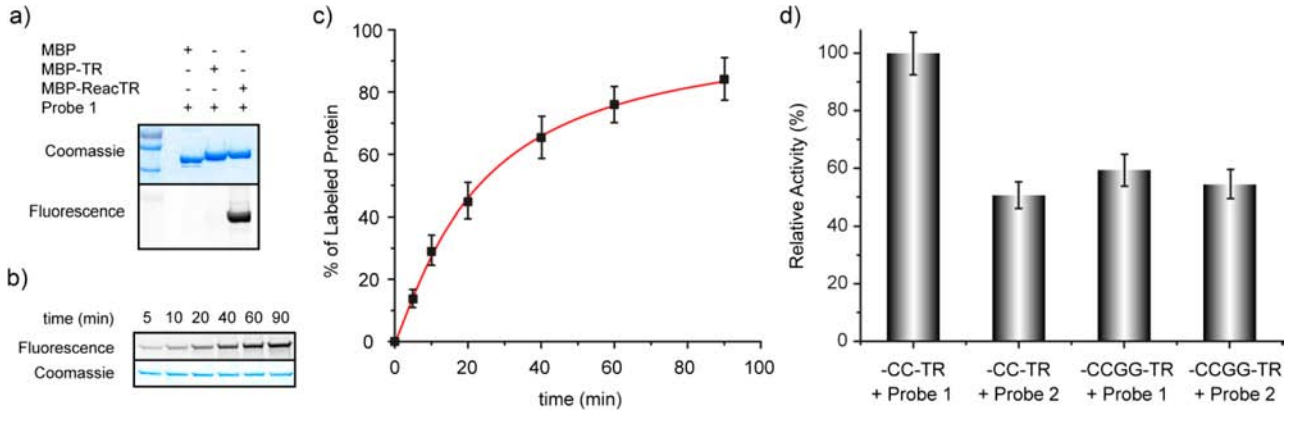

Figure 2. (a) Assessment of the specificity and efficiency of the ReacTR tag based protein labeling method. MBP, MBP-TR, and MBP-ReacTR proteins (5 μM) were incubated with Probe 1 (20 μM) and reaction mixtures were loaded on an SDS-PAGE gel. The fluorescence in the gel was detected, and subsequently, the proteins were stained with Coomassie dye. The first lane shows the protein ladder. (b) Kinetics of the labeling reaction. MBP-ReacTR protein (1 μM) was incubated with Probe 1 (20 μM) and aliquots were taken from the labeling reaction at different time points. Proteins were loaded on the SDS-PAGE gel and in-gel fluorescence was detected. (c) The percentage of the labeled protein was quantified and plotted against time. The yield of the labeled protein was calculated by using a reference protein conjugated with a known equivalent of TexasRed. (d) Relative activities of the -CC-TR (-ReacTR) and -CCGG-TR tags, which are fused to the C-terminus of MBP, against Probe 1 and Probe 2. Reaction between the -CC-TR and Probe 1 was found to be the fastest, and its activity was normalized to 100%.

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Bacteria expressing these three different proteins were incubated with probe 1 (10 μM) at 37 °C. After 1 h, cells were pelleted, washed two times with PBS buffer, and finally analyzed (Figure 3a). Already under a 365 nm UV hand lamp,

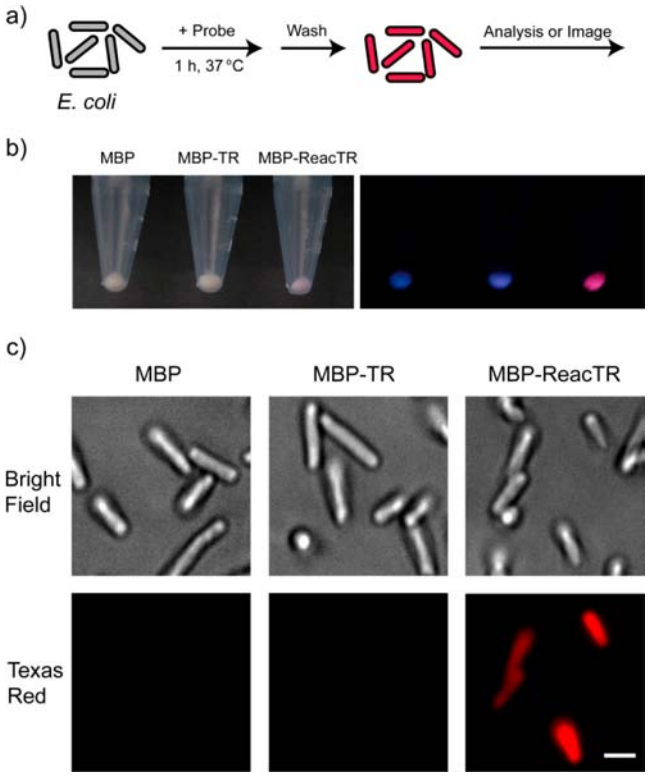

Figure 3. (a) Scheme for the labeling of proteins in live bacteria. (b) Bacteria expressing MBP, MBP-TR, or MBP-ReacTR proteins were incubated with probe 1 and subsequently washed two times with PBS buffer. Pictures of the bacterial pellet in Eppendorf tubes in day light (left) and under a 365 nm UV hand-lamp (right) are shown. (c) Bacteria expressing MBP, MBP-TR, or MBP-ReacTR were treated as described in (b) and then imaged under a fluorescence microscope. Scale bar = 2 μm.

substantial red fluorescence was observed in the pelleted bacteria expressing MBP-ReacTR, whereas no fluorescence was detected in the MBP or MBP-TR expressing bacteria (Figure 3b). We were also able to image single bacteria with fluorescence microscopy and confirmed that bacteria expressing MBP-ReacTR showed significant intracellular fluorescence, whereas the control bacteria expressing MBP or MBP-TR showed very low background fluorescence (Figure 3c). These experiments, first of all, confirmed that the labeling reaction is highly specific and requires the presence of both the TR512 peptide tag and the cysteine residues. We observed only minimal fluorescence background from nonspecific reactions between probe 1 and other cellular entities. Second, these results highlight the advantage of covalent labeling of proteins over noncovalent labeling, as no significant fluorescence was detected in the MBP-TR expressing bacteria, even though MBP-TR protein was reported to bind to the TexasRed fluorophore with high affinity. Most likely, the bound fluorophore is lost during the washing steps, resulting in very low signal-to-noise ratio during fluorescent imaging. It should be noted that the reported binding dissociation constant of 25 pM referred to a multivalent system, namely, the interaction of 5 peptide copies per phage with 5 surface-bound dye molecules. The reported value is therefore an avidity constant, and the K_D for the monovalent interaction (as utilized in our current study) is likely in the μM range.

In conclusion, here we developed a simple one-step method for site-specific covalent labeling of proteins with a TexasRed fluorophore. We utilized a 34-amino-acid TexasRed binding peptide tag (TR512) and attached two cysteine residues to the N-terminus of TR512, yielding the ReacTR tag for covalent labeling of proteins of interest. This small ReacTR peptide tag is genetically encodable and can be fused to a variety of proteins to be imaged. Here, we fused the ReacTR tag to MBP and were able to label MBP in vitro as well as in living bacteria. The ReacTR tag can be considered as complementary to the other existing protein labeling methods and would allow for multiple protein labeling, as it is fully orthogonal to the existing protein labeling methods. The fluorescent probes developed in this study have very simple structures and are cell-permeable, allowing intracellular protein labeling. The probes are also easy to synthesize: they contain only the TexasRed fluorophore and the mildly electrophilic N-α- chloroacetamide group, which selectively reacts with the cysteine group attached to the TR512 tag. Moreover, the labeling reaction between the peptide tag and the TexasRed probe is very fast owing to the proximity effect. Improving the reaction kinetics as well as the binding affinity between the peptide tag and the fluorophore would allow us to label even low-abundance proteins in live cells. However, our method might not be suitable for labeling proteins that reside in oxidative environments, or in situations where a large excess of cysteine thiol groups may generate considerable background signal. The presented method utilizes the direct recognition of the fluorophore by the peptide tag, and does not require any enzyme to transfer the fluorescent moiety onto the peptide tag.[33,34] The extension of this approach to other fluorophores is conceivable and would allow for labeling multiple proteins with different colors. This will, however, require a specific peptide tag for each fluorophore. When combined with the recent advancements in in vitro selection methods, this work may open new avenues for the development of orthogonal reactive peptide tags for a variety of fluorophores spanning a broad range of colors.

■ ASSOCIATED CONTENT

Supporting Information

Materials and instrumentation, experimental procedures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00304.

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Notes

The authors declare no competing financial interest.

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