Steric-Free Bioorthogonal Labeling of Acetylation Substrates Based on a Fluorine-Thiol Displacement Reaction (FTDR)

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Supporting Information

ABSTRACT: We have developed a novel bioorthogonal reaction that can selectively displace fluorine substitutions alpha to amide bonds. This fluorine-thiol displacement reaction (FTDR) allows for fluorinated cofactors or precursors to be utilized as chemical reporters; hijacking acetyltransferase mediated acetylation both in vitro and in live cells, which cannot be achieved with azide- or alkyne- based chemical reporters. The fluorine labels can be further converted to biotin or fluorophore tags using FTDR, enabling the general detection and imaging of acetyl substrates. This strategy may lead to a steric-free labeling platform for substrate proteins, expanding our chemical toolbox for functional annotation of post-translational modifications (PTMs) in a systematic manner.

INTRODUCTION

Bioorthogonal reactions have greatly facilitated protein labeling in complex biological systems and led to widespread applications including imaging, enrichment, and identification, etc.[1-2] Representative bioorthogonal reactions such as azide-alkyne cycloaddition,[3] Staudinger ligation,[4] and tetrazine cycloaddition[2] have resulted in the successful development of chemical reporters, which in conjunction with detection/affinity tags, have advanced our understanding of important biological pathways including PTMs.[5] Yet, current chemical reporters are bulky in length and size, thereby impeding their general applications. Even alkyne or azide- based minimalist reporters for copper-catalyzed azide-alkyne cycloaddition (CuAAC) are still significantly larger than the inherent carbon-hydrogen bond (Figure 1A). This intrinsic steric hindrance has largely limited the application of chemical reporters to metabolic incorporation by enzymes possessing spacious active site pockets. For example, alkyne/azide-labelled PTM precursors or cofactors for acetylation[6-7] and methylation[8-9] were too bulky to be incorporated by many cognate transferases. While "bump-hole" protein engineering strategy could work for a given enzyme, careful balances between mutations, structure folding, and function (potency, selectivity, etc) are required.[10] For many acetyltransferases that function as subunits in protein complexes, mutations in the "hole" may alter their substrate specificities, leading to results different from in vivo sub-acylome,[11] which undermines this approach's broad applications.

Thus, global profiling of PTM substrates such as those of acetylation was usually achieved by antibody-based detections.[12] Yet, the enriched substrates and sites varied significantly per antibody.[12] In our hands, a list of commercially available anti-lysine acetylation antibodies also revealed different sensitivity and substrate specificity (Supplementary Figure S1). Taken together, elucidating the molecular targets of PTMs such as acetylation has been thereby compromised, despite being a key step towards the systematic dissection of PTMs and their roles in biological and pathology-related cellular signaling regulation.[7, 9, 13] To this end, we asked whether a bioorthogonal reaction can be developed to generate reporters for the steric-free labeling of protein substrates and thereby allow for the global profiling of molecular targets.

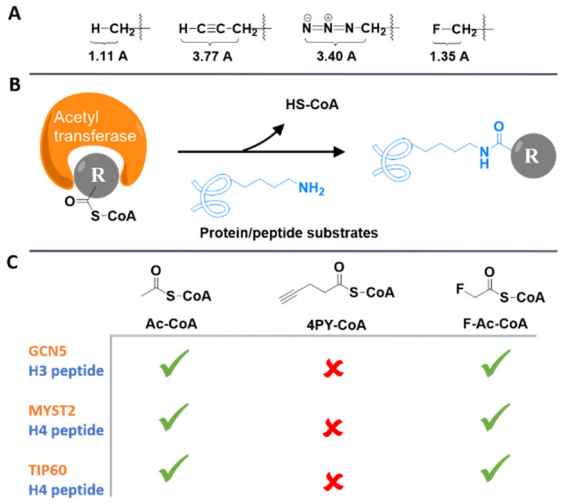


Figure 1. Acetyl-CoA analogs to label KAT substrates through acetyltransferase assay. (A) Bond lengths of C-H, C-C≡CH, C-N_3, and C-F. (B) Illustration of the acetyltransferase assay. (C) Summary of the acetyltransferase assay results with Ac-CoA, 4-pentynoyl (4PY)-CoA, and F-Ac-CoA analogs, respectively.

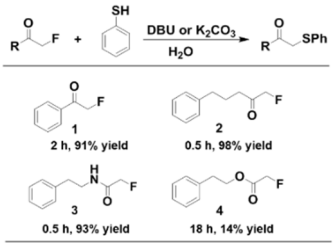
RESULTS AND DISCUSSION

The fluorine atom attracted our interest due to its orthogonality from biological molecules,[14] similarity in size to hydrogen,[14-16] and the close length of the carbon-fluorine bond to the carbon-hydrogen bond (Figure 1A).[16] Fluorinated amino acids have been exploited to label proteins, bringing in minimal perturbations to protein structure and function.[14-15, 17] Using acetylation for a proof of concept, we first wanted to investigate whether fluorinated acetyl-CoA can hijack the CoA metabolism, and be used by acetyltransferases to label their protein substrates (Figure 1B). Despite the in vivo toxicity of its pro-metabolite fluoroacetate,[18] only the late-stage metabolite fluorocitrate was found accountable, mostly damaging organ tissues such as the kidney.[18] Fluorinated acetyl-CoA and fluoroacetate haven't yet been found to inhibit any enzyme, and have been successfully used to study metabolisms.[19-20] Ac-CoA analogs with fluorine (F-Ac-CoA)[21] or alkyne (4-pentynoyl (4PY)-CoA)[7] functional groups were thereby synthesized, and each mixed

with key acetyltransferase GCN5, MYST2, or TIP60 and their corresponding histone peptide substrates.[7, 22-23] Mass spectrometric analysis (Supplementary Figure S2) indicated successful acetyl or acetyl analog labelling after either acetyltransferase was incubated with wild type Ac-CoA or F-Ac-CoA. On the contrary, all acetyltransferases failed to incorporate the alkyne-modified 4-PY-CoA, which is consistent with previous reports.[7, 11] Substitution with the electron-withdrawing fluorine also endowed a much higher hydrolysis rate to F-Ac-CoA, which somehow did not display significantly greater non-enzymatic reactivity (Supplementary Figure S3).[24] Taken together (Figure 1C), fluorine modification afforded a relatively steric-free chemical reporter that can satisfactorily label substrates of acetyltransferases.

Next, we asked whether we can convert the substituted fluorine to other tags such as a fluorescent dye "TAMRA"[25] or a biotin affinity probe[26] for detection, imaging, and future identification of protein substrates. Despite substantial progress in the development of fluorination methodology, there are few efforts on the replacement of fluorine. One most relevant study[27] has incorporated an alpha-fluorinated acetophenone moiety (**1**) into proteins and observed its reaction with a proximal cysteine. This makes us hypothesize that fluorinated acetyl groups may react with thiol derivatives, even at the small molecule level. To test this, we reacted a representative thiol compound, benzenethiol, with a few fluorinated acetyl substrates in water (Scheme 1). In the presence of a strong base, the fluorine alpha to acetophenone (**1**), ketone (**2**), and amide (**3**) were all efficiently displaced (>90% yields). We also observed a similar conversion with the fluoroacetate derivative (**4**) despite a lower yield, which is likely caused by hydrolysis of the ester, given the isolated benzeneethanol side product. The result with **3** particularly encouraged us as it is a model substrate for fluorinated peptides/proteins.

Scheme 1. Exploration of Fluorinated Substrate



To identify the optimal pH range for this reaction, we titrated the pH (Supplementary Figure S4) and found that the reaction rate significantly increased with pH, likely due to the increased deprotonation of benzenethiol. Using mildly basic condition, pH 8.5, in which the reaction has the best reaction rate, we also examined substrate **3**'s reactivity with glutathione and cysteine, both as strong intrinsic nucleophiles that exist in cellular environments (Supplementary Figures S5-S6). Surprisingly, no reaction was observed for both cases upon incubation for 24h, suggesting that substrate **3** could be bioorthogonal to other nucleophilic species. We then investigated substitution effects on benzenethiol in order to efficiently convert fluoroacetamide under the mild physiological reaction condition.[28-29] Exploration of a series of benzenethiol derivatives (**5-13**) found that electron donating groups at the ortho- and para- positions facilitate the reaction by increasing the nucleophilicity (Figure 2, Supplementary S7). Given their theoretical pKa's (< 7.0) (Supplementary Figure S8), almost all of these derivatives should be fully deprotonated. The most reactive derivative, **13**, is a tri-methoxy analog that can reach ~ 81% reaction conversion within 13 hrs. Despite potential issues regarding sterics for the ortho- substitutions, the superior reactivity of **13** in comparison to the meta- substituted derivative **12** indicated that the methoxy groups are not bulky enough to perturb reactivity. Likewise, deriv

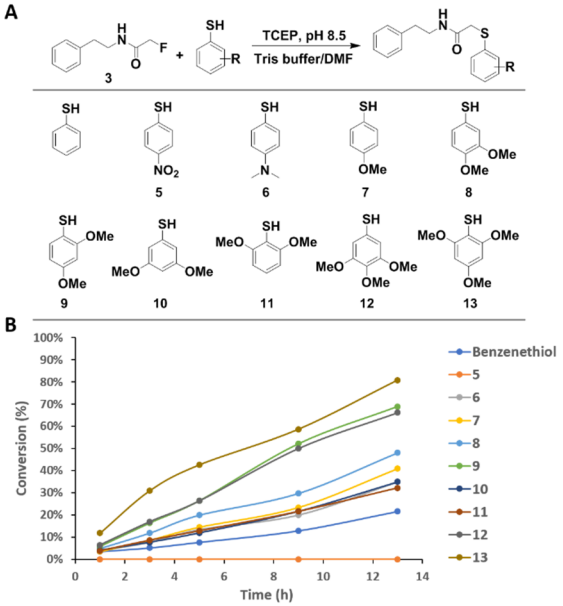


Figure 2. Structure-activity relationship study of benzenethiol derivatives. (A) Scheme of the benzenethiol derivatives. (B) Plot of the conversion against reaction time.

ative **9** that only bears two substitutions at the electron donating sites ended up possessing the same reactivity as **12**. Follow-up kinetics studies based on the reported procedures[30] suggested that the bimolecular reaction between **3** and **13** followed second order kinetics, and had an observed rate constant of $(1.03 \pm 0.06) \times 10^{-3}\ M^{-1}\ S^{-1}$ (Supplementary Figure S9), similar in rates to the classic Staudinger ligation reaction.[30-31] Moreover, the stability of **13** along with substrate **3** were further evaluated in cell lysates (Supplementary Figure S10), and most of them remained intact (61.8% and 100%, respectively). On the contrary, control **3-Cl** possessing the reported chloroacetamide[32] had only 0.15% left, consistent with

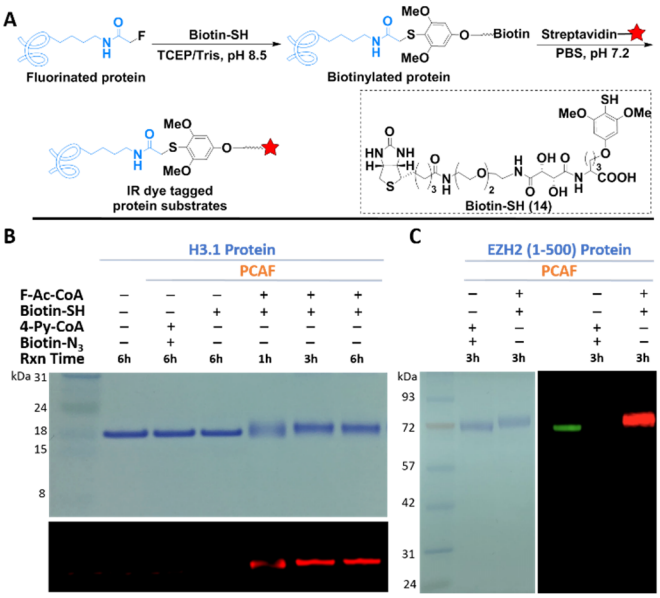


Figure 3. FTDR-based tagging of protein substrates with Biotin-SH probe. (A) Reaction scheme; The red star indicates IR dye. (B-C) Labeling of histone protein H3.1, and nonhistone substrate EZH2 (1-500), respectively; The top panel for (B) and the left panel for (C) are gel images after CBB staining. The other images are for in-gel fluorescent detection of IR dye. The green band in (C) is the reference protein ladder under the 600 nm channel.

previous observations that chloroacetamide readily reacted with cellular proteins.[33] With the aid of LC-MS/MS, the FTDR product of **3** and **13** in cell lysates was also confirmed (Supplementary Figure S11), further corroborating the bioorthogonality of FTDR.

With the most active benzenethiol derivative in hand, we returned to the intriguing question of converting fluorine to functional detection tags such as fluorescent dyes and biotin probes,[26, 34] Thus, we started by constructing a biotin probe (**14**, Biotin-SH) that contains the benzenethiol structure of **13** as the warhead, and the glutamic acid building block (**29**) as the connecting unit to improve overall solubility (Supplementary schemes 7-9). Due to the convenience of ESI-MS characterization on peptides, we first demonstrated the successful conversion of the fluorine label to a biotin tag on the aforementioned histone H3-20 peptide substrate (Supplementary Figure S12). Next, we examined the whole process of labelling and tagging (Figure 3A) on histone protein substrates. Histone H3.1 (Figure 3B) and H4 subunit (Supplementary Figure S13) were each incubated with F-Ac-CoA, and the corresponding KAT under standard in vitro enzymatic reaction conditions.[7] The mixtures were then incubated with the Biotin-SH probe for varied time periods, with subsequent in-gel fluorescent imaging to detect the biotin tagging. For both histone proteins, efficient biotinylation occurred within 1h of incubation, and gradually reached saturation after 3h. Staining with coomassie brilliant blue (CBB) also revealed a complete change of molecular weight, presumably due to the two-step modification. The little signal observed from the control group without prior fluorination also indicated the relative specificity of Biotin-SH. Concurrently, failure of biotinylation was observed for the group using CuAAC labeling, which was consistent with literature reports, and confirmed that many KATs are incapable of uptaking sterically hindered substrates.[7, 11] To check if the fluoroacetyl label could be recognized by histone deacetylases, we also incubated these fluoroacetylated histone proteins with a few reported deacetylases[35] before Biotin-SH tagging. Interestingly, most fluoroacetylation can be efficiently removed (Supplementary Figure S14), suggesting that the label could be substrates for deacetylases as well.

In addition to histones, a growing number of non-histone substrates were recently uncovered, with their roles in protein function regulation being increasingly appreciated.[13] For instance, enhancer of zeste homolog 2 (EZH2) was revealed to be acetylated by PCAF mainly at lysine 348, which improved EZH2's stability and promoted the migration of lung cancer cells.[36] To evaluate whether our labeling strategy is applicable to non-histone substrates, we specifically constructed and expressed the truncated form of EZH2 (1-500) in E. coli, with a shake-flask yield of approximately 2 mg/L. The protein's purity and size were confirmed by SDS-PAGE (Supplementary Figure S15). Reaction of the EZH2 fragment under the same conditions as those for histone proteins resulted in a similar type of tagging as evident by Figure 3C. Taken together, these observations displayed the steric-free in vitro labeling and tagging of a range of known protein substrates using the FTDR reaction, which could not be achieved by the known CuAAC method.

For labeling proteins in living cells, azide or alkyne analogs of fatty acids have been exploited, which were metabolized intracellularly into CoA derivatives.[3-6] To increase their cellular delivery, pro-metabolites with esters masking the polar carboxylate group constituted an effective strategy in recent years.[37] Nevertheless, the intrinsic sterics of these pro-metabolites resulted in varied and suboptimal labeling results,[6, 37] sometimes requiring extensive structural optimization.[37] To explore the utility of our probing system for studying acetylation in the cellular level, we designed the fluorinated version of pro-metabolite, ethyl fluoroacetate (Figure 4A). Given the in vivo toxicity of fluoroacetate,[18] we first evaluated the cell cytotoxicity, and found that this pro-metabolite exhibited minimal toxicity with doses up to 2 mM after 12h of incubation (Supplementary Figure S16). Additional LC-MS/MS studies indeed confirmed its conversion by enzymes to fluoroacetyl-CoA in live cells (Supplementary Figure S17), which, taken together with the observed minimal toxicity in cell lines, could support the applicability of ethyl fluoroacetate and its CoA metabolite to studies in the cell level.

With confidence in the safety profile of our pro-metabolite, we started by treating it with two representative cell lines, HeLa, and

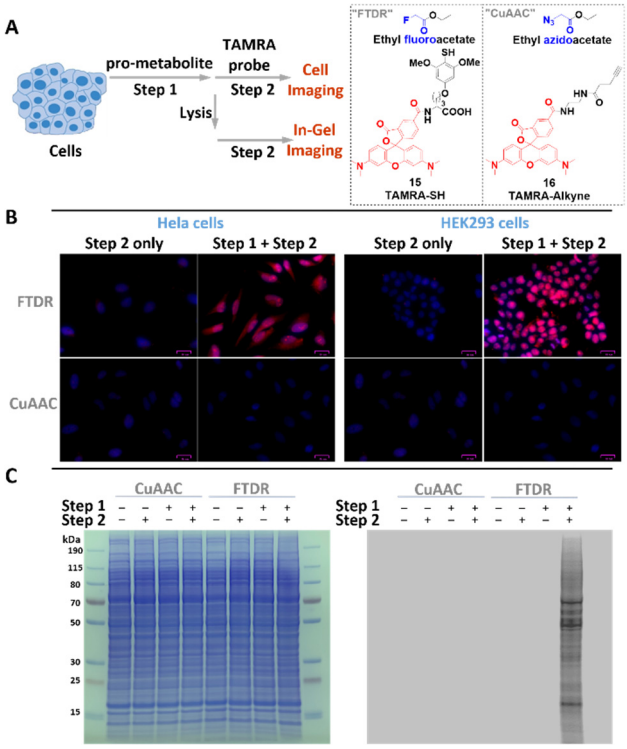


Figure 4. Cellular evaluation of FTDR-based tagging with TAMRA-SH probe. (A) Scheme for cellular pro-metabolite incorporation (1 mM, 6h, at 37 ºC, step 1) and protein substrate detection (step 2). (B) Fluorescent microscopy of fixed and permeabilized cells that were stained by Hoechst 33342 (blue) and TAMRA probes (red); Scale bars: 25 µm. (C) Cell lysate protein labeling by pro-metabolites and detection by TAMRA probes (red); Left panel: PAGE gel stained by CBB; Right panel: In-gel fluorescent detection.

HEK293, followed by subsequent FTDR with the TAMRA-SH probe (**15**) as the second step for fluorescent detection (Figure 4A). Treatment with the azido modified pro-metabolite[37] and the TAMRA-alkyne (**16**) for CuAAC chemistry was performed in parallel as a control, wherein weak signals were previously reported.[37] Direct microscope imaging studies (Figure 4B) revealed much stronger intracellular labeling and tagging with TAMRA following the FTDR based approach, suggesting a drastically more complete profiling of acetylation substrates. We also observed significant fluorescence not only in the nucleus but also cytoplasm, which may indicate the successful labeling of both histones and non-histone proteins. The little background signals emitted from the cells treated with only **15** (step 2) further demonstrated the specificity of the developed -SH probes. Following similar procedures, we also tested the labeling of the cell lysates after metabolic incorporation of fluorine reporters. Lysates following FTDR or CuAAC mediated ligation with TAMRA were separated on SDS-PAGE, and visualized by CBB to confirm the equal amount of protein loading (Figure 4C). Yet, multiple labelled protein bands spanning a wide range of molecular weights were observed only for the lysates of cells that underwent a complete two-step process of FTDR (Figure 4C).

To confirm that the observed protein bands are indeed the substrates of acetylation, we also extracted histones, the major known substrates, from these FTDR-treated cell lysate samples. As shown in Figure 5, histones will be only labelled once the cells have been

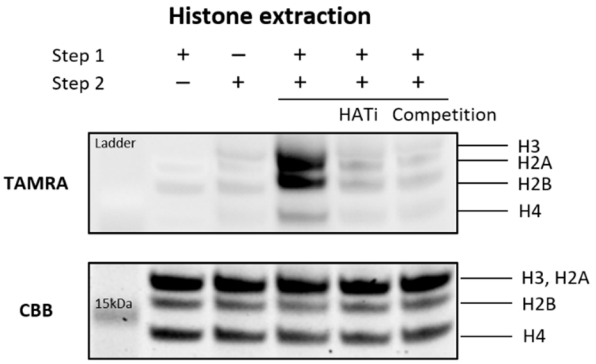


Figure 5. Histone extraction of the HEK293 cell lysates that have been either labelled by ethyl fluoroacetate "pro-metabolite" (step 1 in Figure 4) and/or subsequently the TAMRA-SH probe (step 2 in Figure 4). Top panel: TAMRA fluorescent detection; Bottom panel: CBB staining. HATi: histone acetyltransferase inhibitors; Competition: with ethyl acetate.

subjected to the two sequential steps of treatment. More importantly, we also performed additional control experiments in parallel, and observed diminished TAMRA labeling on histones when the FTDR labeling was either inhibited by histone acetyltransferase inhibitors or competed by over excess amount of ethyl acetate. Taken together, these set of discoveries were consistent with the microscope imaging results, and fully supported that FTDR allowed for profiling the proteome-wide substrates of acetyltransferases from the cellular contexts.

CONCLUSION

In summary, we have developed a fluorine-thiol displacement reaction (FTDR), and used it for steric-free labeling of protein substrates of a representative PTM, acetylation. Along with the benzenethiol derived functional tags, the FTDR-based imaging and detection of substrates demonstrated great potential for globally profiling acetyl transferases' substrates, which are of vital significance for understanding the roles of acetylation in physiology and disease. Although the fluorine tag possesses less steric than the alkyne/azide tags commonly used for CuAAC chemistry, our FTDR rate appeared to be slower and requires mildly basic pH, which could limit certain biological applications such as real-time imaging. Thus, future work would focus on improving and optimizing the FTDR reaction conditions. Nevertheless, this tool kit, together with future applications to quantitative proteomics studies, is expected to offer versatile probes for identifying targets of acetylation, and possibly many other PTMs that are mediated by transferases with restricted active sites.

Supporting Information

Materials and methods, supplementary figures, and NMR spectra are included in the Supplemental Files.

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Notes

The authors declare no competing financial interests.

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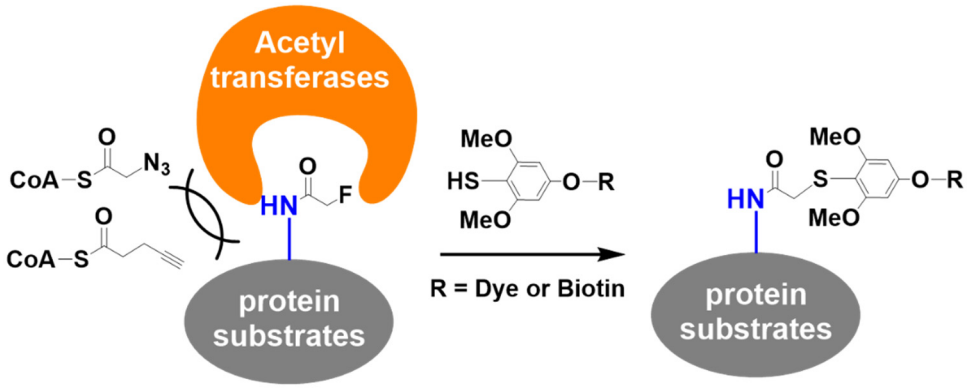
Acetyl transferases
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