Thiomethyltetrazines are Reversible Covalent Cysteine Warheads whose Dynamic Behavior can be "Switched Off" via Bioorthogonal Chemistry Inside Live Cells

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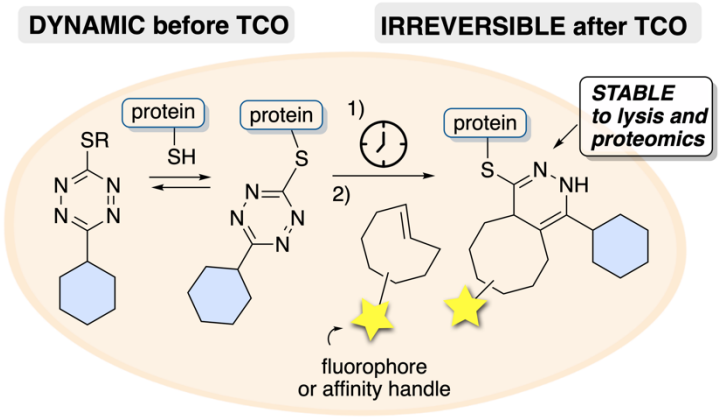
Abstract: Electrophilic small molecules that can reversibly modify proteins are of growing interest in drug discovery. However, the ability to study reversible covalent probes in live cells can be limited by their reversible reactivity after cell lysis and in proteomic workflows, leading to scrambling and signal loss. We describe how thiomethyltetrazines function as reversible covalent warheads for cysteine modification and this dynamic labeling behavior can be "switched off" via bioorthogonal chemistry inside live cells. Simultaneously, the tetrazine serves as a bioorthogonal reporter enabling the introduction of tags for fluorescent imaging or affinity purification. Thiomethyltetrazines can label isolated proteins, proteins in cellular lysates, and proteins in live cells with second-order rate constants spanning two orders of magnitude (k_2 1–100 $M^{-1}s^{-1}$). Reversible modification by thiomethyltetrazines can be switched off upon the addition of *trans*-cyclooctene in live cells, converting the dynamic thiomethyltetrazine tag into a Diels-Alder adduct which is stable to lysis and proteomic workflows. Time-course quenching experiments were used to demonstrate temporal control over electrophilic modification. Moreover, it is shown that "locking in" the tag through Diels-Alder chemistry enables the identification of protein targets that are otherwise lost during sample processing. Three probes were further evaluated to identify unique pathways in a live-cell proteomic study. We anticipate that discovery efforts will be enabled by the trifold function of thiomethyltetrazines as electrophilic warheads, bioorthogonal reporters, and switches for "locking in" stability.

TOC GRAPHIC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INTRODUCTION

Small molecules that can modify protein targets through covalent bond formation represent a growing sector of drug discovery space, and an increasing number of covalent drugs have been approved by the US Food and Drug Administration for therapeutic use.[1-4] Covalent small molecule probes typically target nucleophilic residues for irreversible covalent bond formation to inhibit a target for a therapeutic effect. Relative to non-covalent counterparts, covalent probes can offer advantages of high potency, selectivity and longer time of action,[3] leading to a resurgent interest in targeted covalent probes and the rational design of covalent 'warheads'.[4] Despite the numerous merits of irreversible covalent probes, a concern is that their irreversible nature can result in adverse reactions associated with off-target modification and resulting immunogenicity.[3]

In combination with bioorthogonal chemistry, covalent probes serve as the foundation of activity-based protein profiling (ABPP), a powerful tool for the identification of new drug targets.[5, 6] ABPP is a proteomics technique that has emerged as a tool to identify proteome-wide small molecule-target interactions, by designing an activity-based probe to covalently modify a target or target class. Photoaffinity labeling has been used to extend this strategy to the profiling of non-covalent fragments in live cells.[7] Advances in isobaric labeling have empowered relative quantitative analysis of the proteome.[8-11] Due to the unique reactivity of cysteine within the proteome,[12, 13] a thorough investigation of cysteine reactivity has been explored utilizing proteomics.[14-22] Isotopic Tandem Orthogonal Proteolysis – Activity-Based Protein Profiling (IsoTOP-ABPP) has been developed where an iodoacetamide (IAM) based probe bearing an alkyne tag and isotopically encoded TEV tags has been used to probe the proteome.[16] A competitive isoTOP-ABPP platform was later developed to screen electrophiles. In this platform, a proteome is first treated with an electrophile or a DMSO control. After treatment, both conditions are incubated with an IAM-based probe to alkylate reactive thiols. Protein targets engaged with the electrophile will show reduced alkylation by IAM. A reduction in cysteine alkylation between the control and experimental condition confirms binding of the electrophilic probe.[23] This platform has allowed for the detection of ligandable cysteines for a diverse array of electrophilic fragments.[24-34] Cravatt et. al. have taken advantage of competitive isoTOP-APBB to develop an eloquent workaround for probing the reversible cysteine proteome. After the proteome is labeled with a reversible probe, the proteome is split in half where one portion of the proteome is gel-filtered to remove the reversible probe and the other portion is unfiltered. IAM is sequentially added to broadly react with unlabeled cysteine residues and a reduction in IAM alkylation ratios is used to determine reversible probe binding.[35] As a tool to achieve spatiotemporal control over cysteine labeling, Weerapana et. al. have developed caged alpha-bromomethyl ketone and iodomethylketone probes allowing for time-resolved labeling in the cysteinome with lower cytotoxicity compared to IAM-alkyne probes. [36, 37]

The reversible covalent modification of proteins by small molecules represents an alternative approach to chemical probe discovery. [3, 35, 38-46] Here, covalent bond formation has the advantage of annealability, enabling target engagement to be guided by thermodynamic considerations. The reversible nature of the probe also has the potential to ameliorate concerns about immunogenicity.[46] Such reversible covalent probes (RCPs) draw inspiration from nature's strategy of using reversible disulfide bond formation to control protein structure and function.[46] Ideally, RCPs could be capable of capturing the positive qualities of irreversible probes while reducing concerns about immunogenicity.

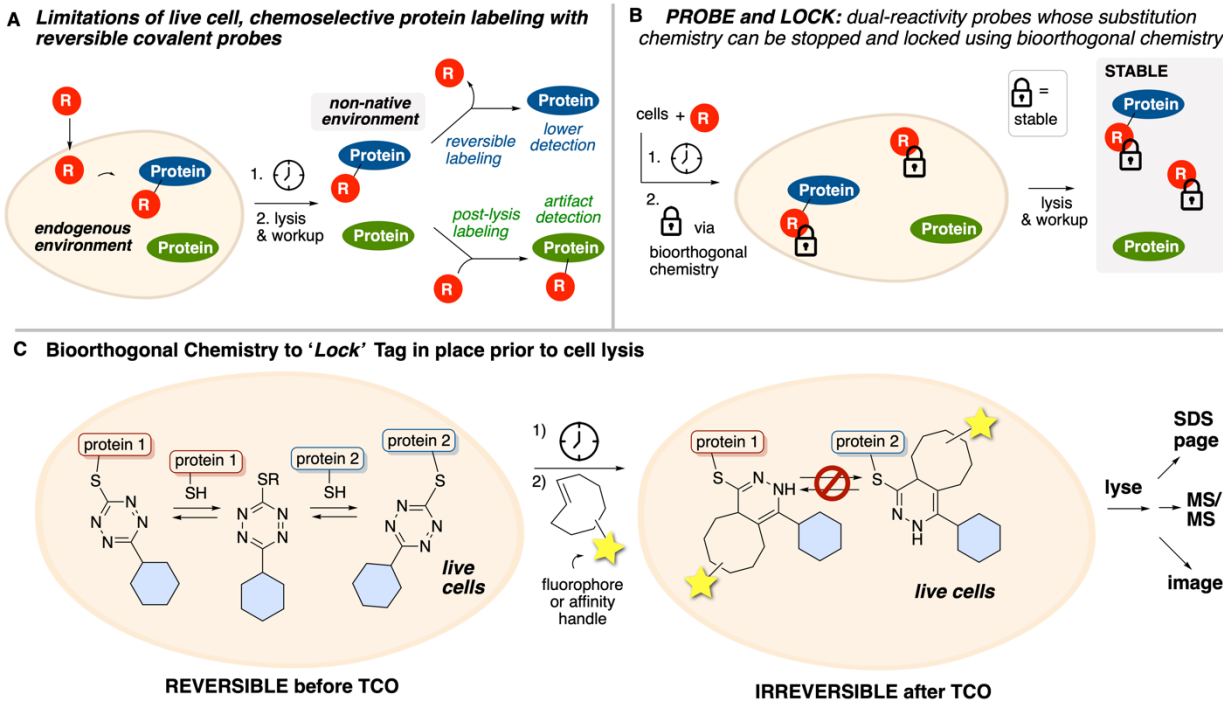
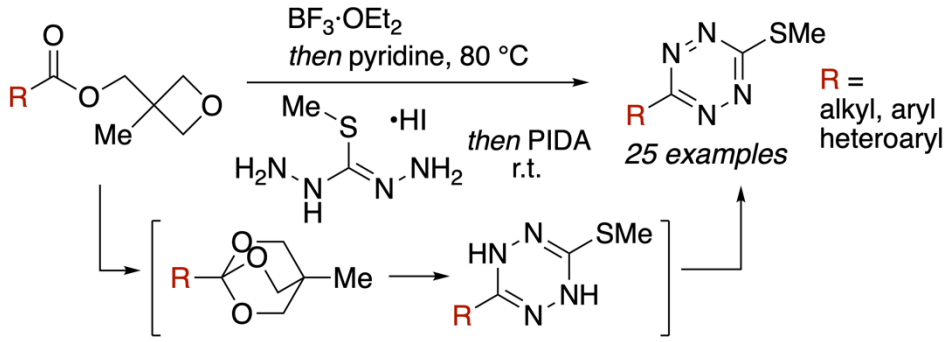


Figure 1: (A) Traditional reversible covalent probes (RCPs) can suffer from scrambling or loss of label during the process of cell lysis and chemoproteomic analysis. (B) Envisioned dual-reactive probes whose substitution chemistry can be quenched and locked in place to detect reversible targets that are otherwise lost during chemoproteomic workflows. (C) Introduced here: Thiomethyltetrazines that can be used as a reversible probe, where after quenching with TCO, a stable tag is produced.

A number of highly selective RCPs have been developed, including several approved drugs.[46] However, tools for the discovery of new RCPs can be limited by their impermanence. RCPs can suffer from scrambling or loss of label during cell lysis, western blot analysis, and proteomic analysis (**Fig 1A**). [38, 40, 41, 47] Strategies for probing reversible covalency have been developed where RCPs are chased with irreversible probes.[23, 35] Additional strategies for the discovery of RCPs in live cells could be highly enabling to the discovery of new biologically active small molecules.

Tetrazine ligation has emerged as a powerful bioorthogonal labeling reaction that is distinguished for its speed, selectivity, and tolerance of a wide range of functional groups.[48-51] Among their numerous applications in chemical biology, tetrazines have been used as bioorthogonal handles in proteomic workflows.[52-57] Recently, the Carrillo group has demonstrated that 3,6-dialkylthiotetrazines can undergo dynamic nucleophilic substitution reactions, and that post-functionalization through Diels-Alder reaction with norbornene gives dihydropyridazine products that no longer exchange.[58, 59] Applications to the syntheses of macrocycles, macromolecular cages, and to polymer chemistry were described, but applications in biology had not been reported.[59] Recently, our group has described a method for preparing thiomethyltetrazines via a one-pot reaction between carboxylic esters and methylthiocarbohydrazide iodide.[60] The method displays a degree of functional group tolerance and can be used to prepare aliphatic, aromatic and heteroaromatic tetrazine thioethers. The tetrazine thioethers prepared by this method serve as a platform for the preparation of mono- and disubstituted tetrazines via Pd-catalyzed reduction (SMe → H) and cross-coupling (SMe → C_{sp2}), respectively (**Scheme 1**).



Scheme 1. One-pot synthesis of functionalized thiomethyltetrazines from ester precursors

Herein, we describe thiomethyltetrazines as a novel class of reversible covalent probe for live-cell protein labeling whose reversible reactivity can be 'switched off' using bioorthogonal chemistry.[61] As illustrated conceptually in **Fig. 1B** and **Fig. 1C**, this new strategy utilizes probes with dual modes of reactivity both as electrophiles and as bioorthogonal reagents. When the probe alone is added to live cells, covalent labeling is reversible. Upon addition of a bioorthogonal *trans*-cyclooctene (TCO), the tetrazine is rapidly converted into a dihydropyridazine product which is unable to participate in subsequent S_NAr chemistry, thereby locking the tag as a stable conjugate *inside live cells prior to cell lysis and proteomic workup* which enables the detection of targets that may otherwise be lost during western blot and proteomic workflows. Taken together, the small thiomethyltetrazine serves three distinct functions as (1) a reversible covalent S_NAr warhead for cysteine, (2) a bioorthogonal group that through reaction with TCO can be "switched off" reversibility to provide a stable conjugate and (3) a handle for introducing fluorescent or affinity tags through the same bioorthogonal reaction with TCO. We demonstrate reversible covalent labeling at the small molecule and protein level, and evaluate labeling across a panel of eight distinct probes with kinetics that can be tuned over two orders of magnitude (k_2 up to 10^2 $M^{-1}s^{-1}$). Tetrazine exchange chemistry via S_NAr can be switched off through rapid reaction

with TCO derivatives that enable live cell imaging, isolation, SDS-PAGE/western analysis, and site identification and quantification by proteomic analysis. Proteomic studies show how "locking" the probe prior to cell lysis is necessary to preserve labeling, and a study of three different thiomethyltetrazine derivatives shows unique pathways for the top 20 proteins modified by each probe.

RESULTS AND DISCUSSION

Confirming thiomethyltetrazine labeling by in vitro protein labeling

Initially, screening was conducted to test if thiomethyltetrazines can undergo nucleophilic aromatic substitution (S_NAr) chemistry with nucleophilic amino acids in PBS buffered aqueous solution. 3-Thiomethyl-6-(4-pyridyl)tetrazine (**A**) was prepared as described previously.[60] Probe **A** (50 μM) was incubated with either Histidine, Lysine, Serine, Threonine, Glutamate or *N*-acetylcysteamine (**N-AcCys**) (1 mM) for 1.5 hours at room temperature (**Fig. 2A**). Only **N-AcCys** was labeled by **A** to give **1** in 60% yield (**Fig. 2B**). The conversion of **A** is relatively high considering that tetrazines can be competitively reduced to an S_NAr-inactive dihydrotetrazine in the presence of mM thiol. Labeling was not observed on any other amino acids screened (**Fig. S2**), suggesting the labeling is thiol-specific.

To confirm that thiomethyltetrazines can label cysteine on a protein, LC-MS studies were conducted (**Fig. 2C-F**). Thiomethyltetrazine **A** (100 μM) was incubated with green fluorescent protein (**GFP-SH**, 25 μM) for 60 minutes. As indicated by the deconvoluted mass spectral data, a mass shift corresponding to S_NAr chemistry between **A** and **GFP-SH** to yield **GFP-STz** was observed confirming thiomethyltetrazine's ability to label a cysteine-containing protein (**Fig. 2E**). Upon addition of *ax*-5-hydroxy-*trans*-cyclooctene (***ax*-5'OH-TCO**, 300 μM) to **GFP-STz**, mass spectrometry confirmed that a reaction had proceeded to give the Diels-Alder adduct **GFP-DA** (**Fig. 2F**). Furthermore, **GFP-DA** was stable for up to 96 hours in aqueous buffer (**Fig. S4**). We note that, as in the labeling of *N*-acetylcysteine, the labeling of GFP does not go to completion, likely due to tetrazine reduction and the concomitant formation of **GFP-SS-GFP** dimer, which cannot be discerned from **GFP-SH** under the mass spectrometry conditions. To further demonstrate the thiol-specificity of thiomethyltetrazine labeling, **GFP-SH** was alkylated with IAM to cap solvent-exposed cysteines prior to labeling. No labeling was observed when GFP capped with IAM was incubated with **A** confirming that thiomethyltetrazine labeling is thiol-specific (**Fig S5**).

Next, second-order rate constants (k_2) were determined for probes **A, B,** and **C.** Previous reports have shown that tetrazines have a unique ability to act as fluorescence quenchers when bound to fluorophores,[62] and as such, GFP fluorescence is quenched when bound to tetrazine.[63, 64] Therefore, we predicted that thiomethyltetrazines can quench the fluorescence of **GFP-SH** when S_NAr chemistry has taken place on cysteine. Furthermore, we can determine k_{obs} by monitoring the decrease in **GFP-SH** fluorescence over time at different tetrazine concentrations to calculate the biomolecular rate constant (k_2) of thiomethyltetrazine labeling on a purified protein. When **GFP-SH** (10 μM) was incubated with **A** (100 μM), a fluorescence decay was observed over time

confirming that **GFP-SH** fluorescence is partly quenched (by 28%) upon S_NAr chemistry to form **GFP-STz** (**Fig. S7A**). Monitoring GFP fluorescence quenching to calculate k_2 was repeated for probes **B** and **C** (**Fig. S8-S9**). Thiomethyltetrazine reactivity spanned two orders of magnitude for these probes, with k_2 =103 ± 3 $M^{-1}s^{-1}$ for **A**, k_2 =1.10 ± 0.05 $M^{-1}s^{-1}$ for **B**, and k_2 =3.61 ± 0.24 $M^{-1}s^{-1}$ for **C** (**Fig. 2G**). No fluorescence decrease was observed for the control when **GFP-SH** (10 μM) was incubated with **MeTzPh** (100 μM), confirming the SMe electrophile must be present for S_NAr chemistry to take place and for fluorescence quenching to occur (**Fig. S7B**).

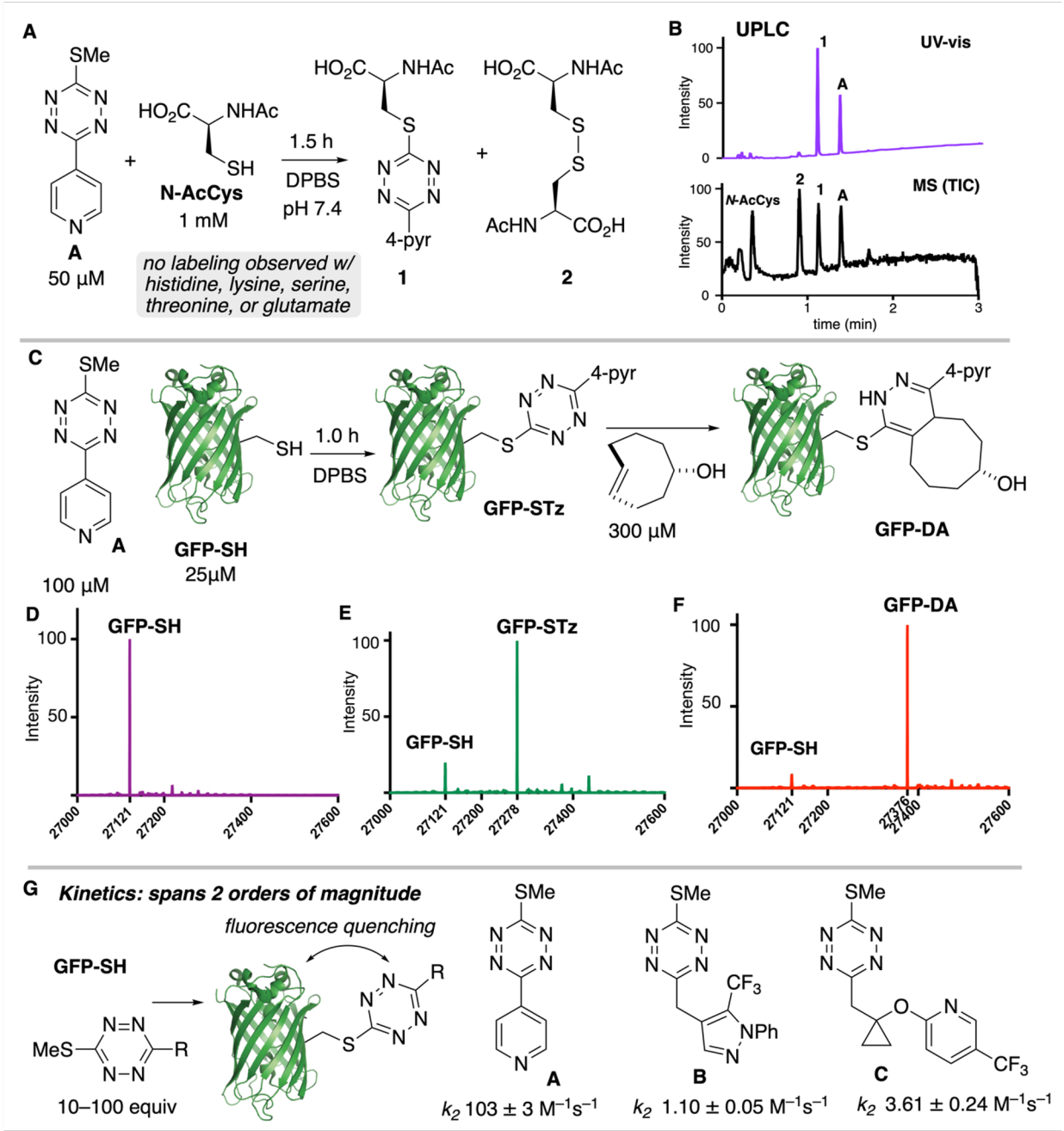


Figure 2. (A) Amino acid screening with **A**. (B) UPLC (UV and MS channels) for **A** + **N-AcCys** to form **1**. (C) Intact protein labeling, **GFP-SH** was incubated with **A** to form **GFP-STz** and ***ax*-5'OH-TCO** was added to form **GFP-DA**. (D) Deconvoluted ESI MS of **GFP-SH**. (E) Deconvoluted ESI MS of **GFP-STz**. (F) Deconvoluted ESI MS of **GFP-DA**. (G) Fluorescence quenching of GFP by **A** to determine second order rate constants.

"Switching-off" reversible covalent chemistry through bioorthogonal reactivity

We anticipated that proteins would undergo reversible electrophilic modification by thiomethyltetrazines at accessible cysteine sites, and that the reversibility of the electrophilic modification would cease when **GFP-STz** is converted to **GFP-DA** upon Diels-Alder reaction with TCO (**Fig. 3A**). Previously, our group demonstrated bioorthogonal chemistry could be used as a tool to exercise temporal control over sulfenic acid labeling in live cells.[65] With the thiomethyltetrazine warhead, we sought to use bioorthogonal chemistry to give us the unique ability to "switch-off" reversible labeling to provide stable conjugate tags that facilitate visualization or isolation.

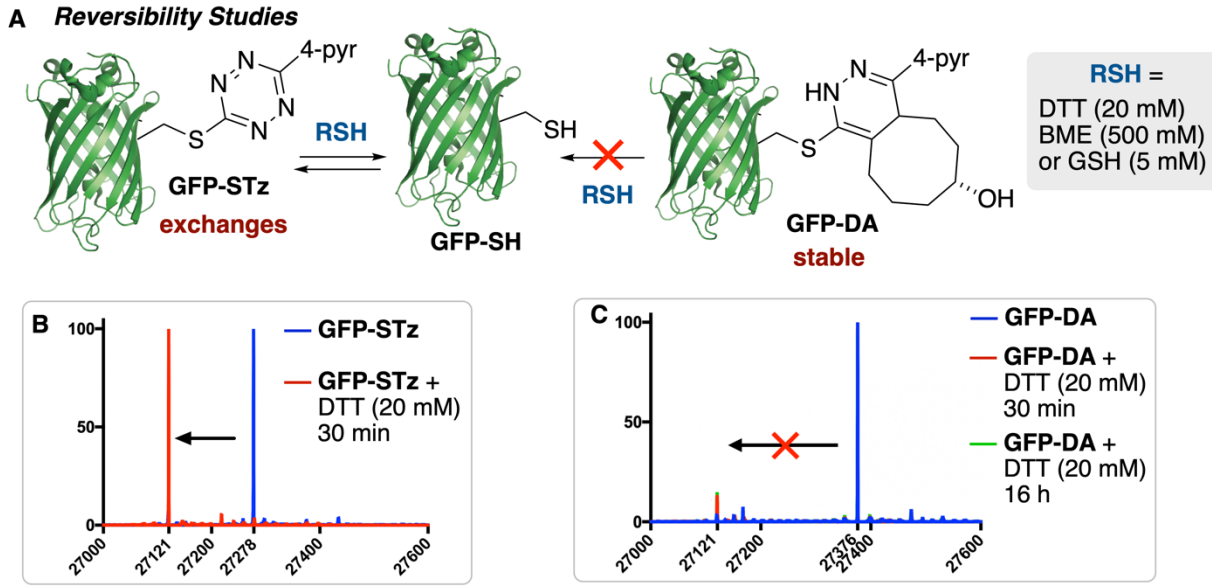


Figure 3: (A) **GFP-STz** is reversible in presence of thiols while **GFP-DA** is stable in the presence of thiols. (B) Deconvoluted ESI MS of **GFP-STz** incubated with 20 mM DTT at time =0 and time=30 min (C) Deconvoluted ESI MS of **GFP-DA** incubated with 20 mM DTT at time=0, time=30min, and time=16 hour.

To study the reversibility of thiomethyltetrazine labeling on a protein level, **GFP-SH** (10 μM) was labeled with **A** (100 μM) for 30 minutes at room temperature to give **GFP-STz**. After complete labeling, 20 mM dithiothreitol (DTT), a reducing agent commonly used in proteomic workflows, was added to **GFP-STz** and incubated for an additional 30 minutes. By MS, a complete loss in labeling is observed due to the reversible sulfide exchange between **A** and DTT, confirming the reversibility of thiomethyltetrazine labeling (**Fig. 3B**). To study if reversibility over electrophilic modification could be stopped and "locked-in", **GFP-SH** (10 μM) was labeled with **A** (100 μM) and ***ax*-5'OH-TCO** (300 μM) was added to give **GFP-DA** along with a small signal assigned to **GFP-SS-GFP**. After complete conversion to **GFP-DA**, 20 mM DTT was added. After the initial incubation with DTT, there is a slight recovery of signal which we assign to reduction of **GFP-SS-GFP** to **GFP-SH**, which is more readily ionized. However, that recovery stalls after 30 minutes, and **GFP-DA** is stable in the presence of DTT

for up to 16 hours (**Fig. 3C**). Thus, labeling loss was not observed confirming that **GFP-DA** is stable in the presence of other thiols. Similar results were observed when these experiments were repeated with glutathione (5 mM) and ß-mercaptoethanol (BME, 500 mM) (**Fig. S10**). These results demonstrate that reversible electrophilic protein modification by thiomethyltetrazine can be stopped, or "switched-off", and that the label is subsequently preserved.

Taking these results into consideration, we envisioned thiomethyltetrazines could be used as a unique proteomics tool to probe reversible covalency in live cells. To analyze protein targets by proteomic analysis, reversible electrophilic modification by thiomethyltetrazines would be quenched with the addition of TCO, creating a stable tag for bottom-up proteomics analysis. This tool would allow for the detection of protein targets labeled with RCPs that would be 'lost' when tag-labeled proteins undergo denaturing conditions and proteolytic digestion.

Probing cell lysates with a fragment library of thiomethyltetrazines

We next demonstrated the ability of thiomethyltetrazines to label proteins in cell lysates. A fragment library of thiomethyltetrazines (**A-H**) was readily prepared and used to probe HeLa cell lysates [60]. Compounds **A-H** (100 μM) were incubated with proteins in HeLa cell lysates for 45 minutes. Next, proteins that were labeled were fluorescently tagged with **a-TCO-TAMRA** (350 μM) (**Fig. 4A, B**). Different labeling patterns for probes **A-H** were observed, demonstrating the ability for thiomethyltetrazine reactivity to be tuned (**Fig. 4C**). In general, 3-aryl-6-thiomethyltetrazines (**A, G, H**) tended to have higher reactivity, with probe **A** being the most reactive. This observation is likely due to the increased electrophilicity of these probes by attaching electron withdrawing groups to the tetrazine core.

The scope of thiomethyltetrazine probes was expanded to include fluorescent thiomethyltetrazine derivatives. Proteins in HeLa cell lysate were labeled with a fluorogenic thiomethyltetrazine-BODIPY (**BODIPY-TzSMe**)[60] (1 μM -100 μM) for 45 minutes. ***ax*-5'OH-TCO** (400 μM) was added and fluorescently tagged proteins were analyzed by in-gel fluorescence. Dose-dependent labeling of fluorescent proteins was observed confirming that the tool box of thiomethyltetrazine probes can be extended to include fluorescent derivatives (**Fig. 4D**). When the labeling was repeated with **BODIPY-Tz** [66, 67] a tetrazine-BODIPY probe lacking the thiomethyltetrazine electrophile, no labeling was observed demonstrating the importance of the electrophilic SMe-group (**Fig. 4E**). Different labeling

patterns were also observed when comparing protein labeling by **A** and **BODIPY-TzSMe**, further demonstrating the utility and tunability for these thiomethyltetrazine probes (**Fig. S12**).

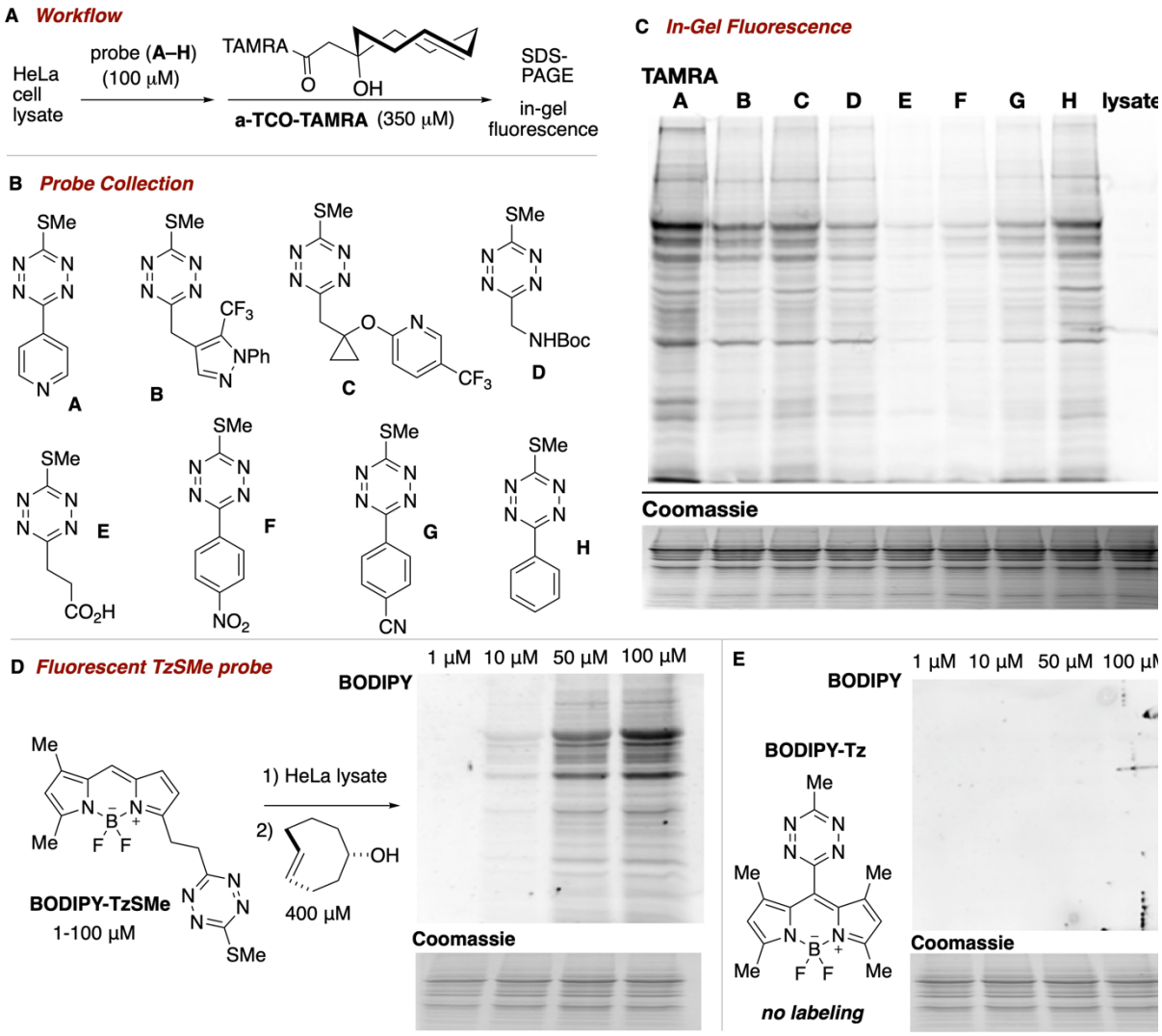


Figure 4. (A) General workflow for labeling proteins in HeLa cell lysate with thiomethyltetrazine fragment library. (B) Fragment library of thiomethyltetrazines. (C) Comparing cell lysate labeling by thiomethyltetrazine library. (D) Probing cell lysates with **BODIPY-TzSMe**. (E) Probing cell lysates with **BODIPY-Tz** as a control.

Live-cell labeling and imaging with thiomethyltetrazine

Next, we demonstrated the ability of thiomethyltetrazines to label proteins in their native state in live cells. HeLa cells were incubated with **A** (50 μM) for 5 minutes in DPBS. After extensive washing, **SiR-aTCO** (500 nM) was added and cells were incubated for an additional 30 minutes. The addition of **SiR-aTCO** quenches the labeling and fluorescently tags proteins labeled by **A.** Cells were extensively washed to remove any unbound **SiR-aTCO** and cells were imaged live using confocal microscopy (**Fig. 5A**).

Silarhodamine fluorescence was only detected in the conditions where both **A** and **SiR-aTCO** were added, confirming that thiomethyltetrazines can label cysteines under physiological conditions (**Fig. 5B**). Thiomethyltetrazine labeling is observed throughout the entire cell with a significant level of nuclear labeling. Importantly, silarhodamine fluorescence was not detected in any of the controls, further demonstrating that thiomethyltetrazine electrophile is required for labeling (**Fig. 5, Fig. S14**).

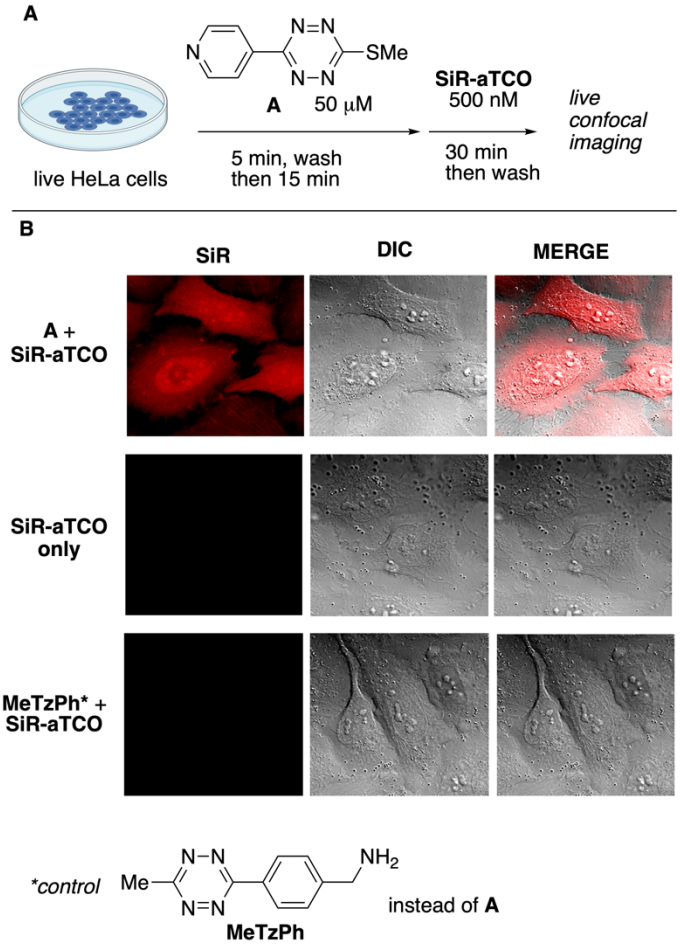


Figure 5. (A) General workflow for live-cell imaging with **A.** (B) Confocal live-cell imaging with **A** and **SiR-aTCO** compared to controls.

Live-cell labeling with time-course quenching and thiomethyltetrazine dose dependence

In live cell experiments, time-course experiments were carried out with thiomethyltetrazine probe **A**, whereby bioorthogonal quenching with TCO was conducted to exercise temporal control over labeling. In order to profile the proteome with thiomethyltetrazines, a TCO bound to a desthiobiotin moiety (**aTCO-d-biotin**) was utilized. Desthiobiotin has been shown to result in increased coverage for probe labeled peptides in comparison to biotin.[22,68] Live HeLa cells were incubated with **A** (25 μM) and labeling was quenched at 0, 1, 5, 10, and 20 minutes by the addition of **aTCO-d-biotin** (75 μM). The "t = 0" timepoint reflects premixing. Cells were washed and **MeTzPh** (100 μM, structure in Fig 5) was

added to quench TCO and validate that **aTCO-d-biotin** was cell permeable. Cells were lysed and analyzed by western blot analysis (**Fig 6A**). A time-course gradient of labeling is observed demonstrating temporal control over electrophilic modification by **A** by the addition of TCO (**Fig. 6B**). Dose-dependent labeling was observed when a concentration gradient of **A** (1 μM–50 μM) was incubated with live HeLa cells for 20 minutes in DPBS. The labeling was 'stopped and locked' upon the addition of **aTCO-d-biotin** (in 3-fold excess) and cells were lysed for western blot analysis (**Fig. 6C**).

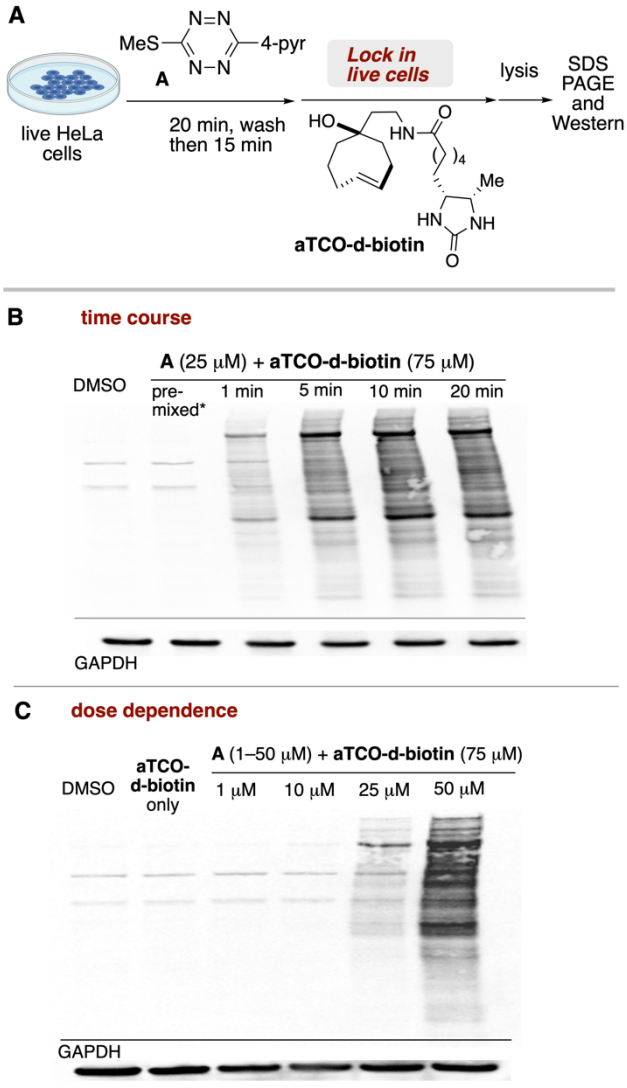


Figure 6. (A) General workflow for live-cell labeling with western blot analysis. Desthiobiotinylated proteins were detected using HRP-streptavidin. (B) Labeling proteins in live cells with **A** and time course quenching with **aTCO-d-biotin**. For the premixed conditions, **A** and **aTCO-d-biotin** were incubated for 30 minutes prior to incubation with cells. (C) Dose dependence of labeling with **A**.

"Switching off" dynamic labeling behavior enables proteomic analysis of RCPs

Taunton et. al. have shown that tag-loss occurs when proteins labeled with RCP's undergo proteolytic digestion, resulting in no modified peptides. [38, 40, 41] We have shown that thiomethyltetrazine labeling is reversible and that upon addition of TCO, Diels-Alder reaction converts the thiomethyltetrazine to a stable tag (**Fig 3**). Therefore, we envisioned that thiomethyltetrazines could be used as a unique proteomics tool to profile RCPs for proteomic analysis and for downstream discovery efforts.

For proteomic studies, live HeLa cells were incubated with **A** (50 μM) for 20 minutes in DPBS. Adding **aTCO-d-biotin** (150 μM) at different points within the proteomic workflow allowed us to analyze the importance of locking the label within live cells: (**I**) adding the **aTCO-d-biotin** prior to cell lysis is expected to 'lock' the label in live cells, (**II**) addition of **aTCO-d-biotin** after cell lysis, (**III**) addition of **aTCO-d-biotin** after cell lysis, reduction of proteins with DTT, and alkylation of free thiols with IAM (**Fig. 7A**). For all conditions, after incubating with **aTCO-d-biotin** for 30 minutes, the TCO was quenched with **MeTzPh** (100 μM, structure shown in Fig. 5). By western blot analysis, different labeling patterns are observed when reversible thiomethyltetrazine labeling is quenched along different time points of a proteomic workflow (**Fig. 7B**). The highest amount of labeling is observed when the labeling is 'stopped-and-locked' in live cells (**I**). A labeling decrease is observed for conditions where **aTCO-d-biotin** is added after lysis and workup (**II** and **III**). These results demonstrate the importance to 'stop-and-lock' RCP labeling while they are still inside live cells, as RCPs can scramble resulting in loss of tag during cellular lysis and proteomic workups. This experiment was repeated with two different cellular lysis conditions to confirm that loss of labeling is not due to conditions used for cellular lysis (**Fig. S16**). Dramatic differences in labeling patterns are also observed when comparing thiomethyltetrazine labeling in live cells to labeling in cellular lysates (**Fig. S17**). This observation is due to the thiomethyltetrazine tag being able to label proteins in cell lysates that may not be accessible in live cells, further demonstrating the importance to 'stop-and-lock' live-cell labeling with RCPs.

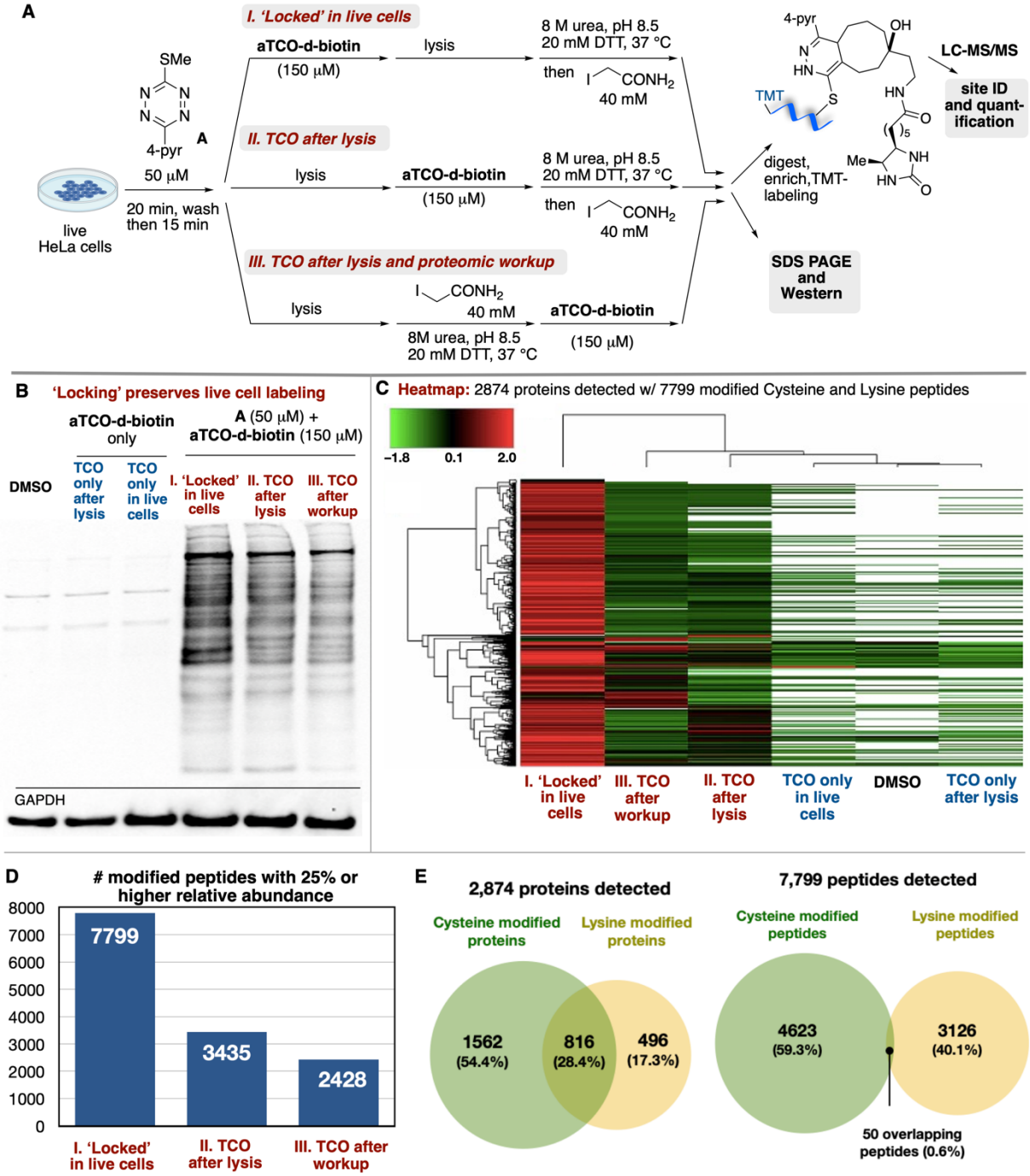


Figure 7. (A) General workflow for quenching labeling by **A** at various points along a proteomics workflow for western blot and bottom-up proteomics analysis. (B) Western blot analysis for the different quenching conditions depicted. Desthiobiotinylated proteins were detected using HRP-streptavidin. (C) Heatmap of protein abundances for the different quenching conditions depicted. (D) Relative peptide abundances were calculated as a percentage of **I**. *'Locked' in live cells* conditions and any peptides below 25% relative abundance were filtered out. (E) Comparing cysteine and lysine modified proteins and peptides. Modified peptides were mapped back to their proteins and analyzed using a Venn diagram.

For proteomic comparison of conditions **I**-**III** in Fig. 7, the same initial workflows were followed. After labeling, proteins were digested and modified peptides were enriched with streptavidin-affinity chromatography. After extensive washing, modified peptides were eluted from streptavidin beads with acidic conditions and labeled with tandem mass tags (TMT) for quantification: 2874 modified proteins were detected with 7799 modified cysteine and lysine peptides. No thiomethyltetrazine modification was observed on glutamate, arginine, serine, threonine, or histidine. Similar results to the western blot analysis, adding **aTCO-d-biotin** in live cells to 'stop-and-lock' labeling before cellular lysis and proteomic workup (**I**) resulted in highly abundant modified proteins and peptides, whereas all other conditions resulted in major decreases in protein and peptide abundances (**Fig. 7C**). A greater than 50% reduction in modified peptides is observed in conditions **II** and **III** when filtering out modified peptides with a lower than 25% relative abundance (**Fig. 7D**). Thus, in experiments where reversible labeling is not switched-off prior to lysis, an inaccurate profiling of the proteome can occur where some protein targets may even be lost during proteomic workflows. To get the most accurate depiction of proteomic targets for thiomethyltetrazine probes, TCO can be added to turn-off the reversible electrophilic modification by thiomethyltetrazines and create a stable tag for target identification by proteomic analysis.

It is important to note the 20% coverage increase by lysine-modified proteins (**Fig. 7E**). As we have demonstrated that in-vitro thiomethyltetrazine labeling is thiol specific, we suspect this increased coverage is largely due to thiomethyltetrazines undergoing S-to-N shift, a phenomenon well known in acyl transfer reactions in proteins.[69] The lack of doubly modified peptides further supports this hypothesis, as the S-to-N shift mechanism requires labeling on cysteine prior to transfer to lysine. These results suggest that the thiomethyltetrazine toolbox can be expanded to target lysine residues via proximity labeling.

Fragments bearing thiomethyltetrazine warheads target specific protein classes

Tuning the reactivity for cysteine targeting probes have been previously reported. [20, 21, 24, 25, 32, 34, 70, 71] Thiomethyltetrazine reactivity can be tuned based on modulating the R-group attached to the thiomethyltetrazine core (**Fig. 2G, Fig. 4**). To determine if different thiomethyltetrazines can target different proteins in live cells, probes **A**, **B**, and **C** (50 μM) were incubated with live HeLa cells for 20 minutes in DPBS. **aTCO-d-biotin** (150 μM) was added to quench the labeling and after 30 minutes **MeTzPh** (100 μM) was added to quench TCO (**Fig. 8A**). Cells were lysed and protein labeling was analyzed by western blot. Probe **A** ($k_2 = 103 \pm 3\ M^{-1}s^{-1}$) was the most reactive probe and targeted the greatest number of proteins within the proteome while probes **B** ($k_2 = 1.10 \pm 0.05\ M^{-1}s^{-1}$) and **C** ($k_2 = 3.61 \pm 0.24\ M^{-1}s^{-1}$) were less reactive (**Fig. 8B**). Probe **C** selectively labeled a handful of proteins while probe **A** was more promiscuous in labeling.

To determine protein targets of **A**, **B**, and **C**, proteomic workup and analysis was performed as described above. 1298 proteins were detected with 1940 modified cysteine and lysine peptides. Similar results to

the western blot analysis were obtained where targeting the proteome with probe **A** gave a higher number of modified proteins and peptides compared to the other probes (**Fig. 8C**). Furthermore, probe **A** selectively labeled 221 proteins (**Fig. S22**). To compare protein targets of the different probes, the top 20 proteins modified by **A**, **B**, and **C** were compared (**Fig. 8D**)**.** Probe **B** had unique targets, whereas **A** and **C** had partial overlap with 9 out of 20 top proteins coinciding. To compare the unique pathways of the proteins targeted by **A**, **B**, and **C,** enrichment analysis was performed with Enrich r (**Table S6-S8**). Thiomethyltetrazine probes had a high propensity to label nuclear proteins, a result consistent with live-cell imaging (**Fig. 5**). Uniquely, probe **A** targeted proteins localized in intracellular non-membrane bounded organelles with protein targets involved in purine ribonucleoside triphosphate binding. Probe **B** uniquely targeted proteins involved in cell cycle regulation while probe **C** uniquely targeted proteins localized to the intracellular organelle lumen with proteins involved in protein localization to organelles (**Fig. 8D**). These results demonstrate how modulating the thiomethyltetrazines core can result in probes with different selectivity and reactivity. The unique proteins and pathways identified by probes **A**, **B**, and **C**, suggest that thiomethyltetrazines can be tuned to target a specific class of proteins.

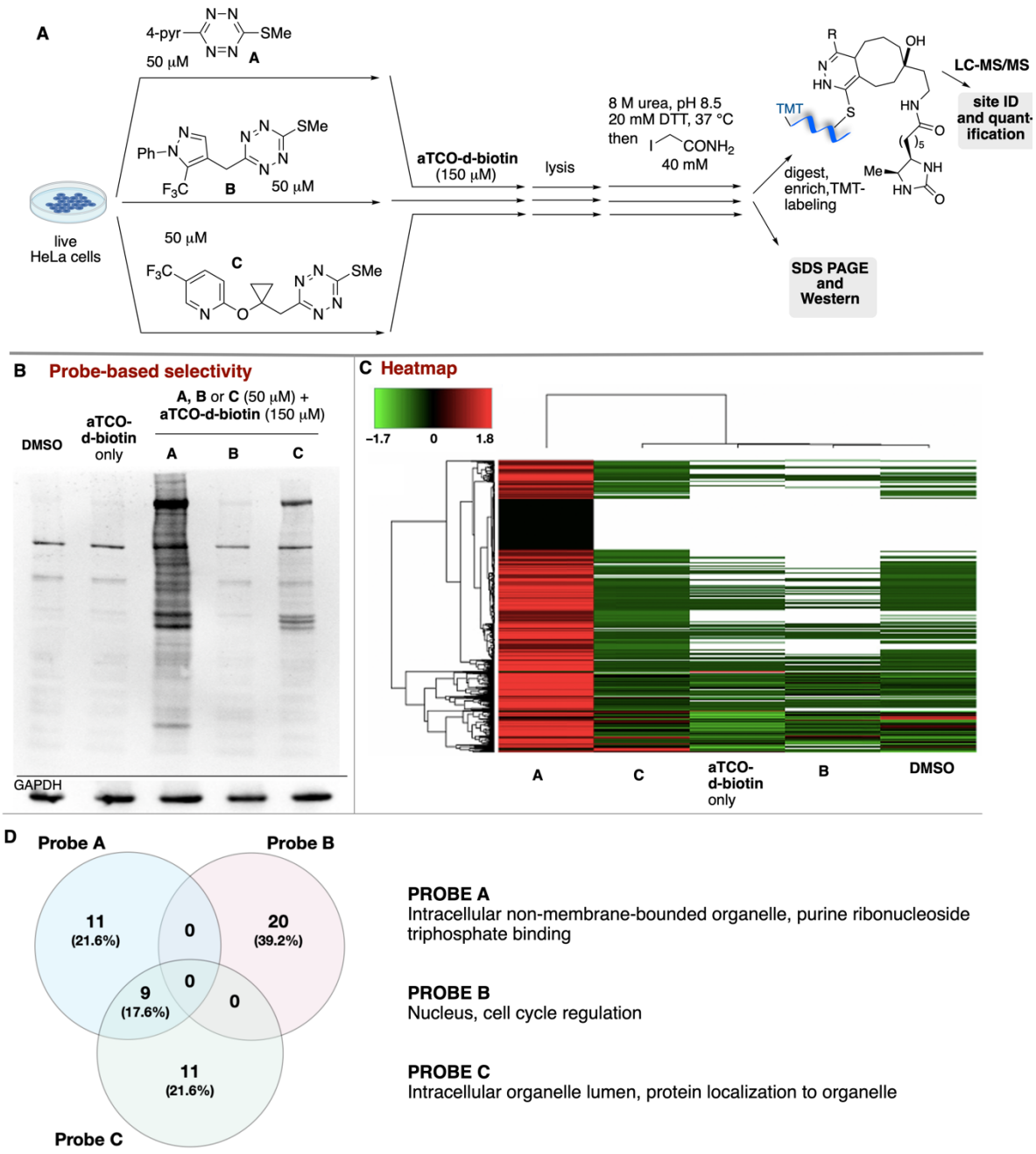


Figure 8: (A) General scheme for labeling HeLa cell proteome with **A**, **B**, and **C** and analyzing by western blot and proteomic analysis. (B) Western blot analysis of the proteome labeled with **A**, **B**, and **C**. Desthiobiotinylated proteins were detected using HRP-streptavidin. (C) Heatmap of protein abundance for proteomes labeled with **A**, **B**, and **C**. (D) Unique pathways of the top 20 proteins modified by probes **A**, **B**, and **C** using Enrich r. Top 20 protein targets were chosen based on the top 20 highest average raw abundance for modified peptides with a 1.5-fold or greater abundance over control conditions.

CONCLUSIONS

We describe how thiomethyltetrazines can function as reversible covalent warheads for cysteine modification whose reversible labeling behavior can be "switched off" via bioorthogonal chemistry inside live cells. The tetrazine also serves as bioorthogonal reporter that enables the introduction of tags for fluorescent imaging or affinity purification. Thiomethyltetrazines have second-order rate constants (k_2 = 1–100 $M^{-1}s^{-1}$) that span two orders of magnitude and are capable of labeling isolated proteins, proteins in cellular lysates, and proteins in living cells. When *trans*-cyclooctene is added to live cells, reversible modification by thiomethyletrazines can be turned off, transforming the reversible thiomethyltetrazine tag into a Diels-Alder compound that is resistant to proteomic processes and lysis. To show temporal control over electrophilic labeling by thiomethyltetrazines, time-course studies were employed. It is demonstrated that protein targets that would otherwise be lost during sample processing with RCPs, can be identified by "locking in" the tag using Diels-Alder chemistry. A panel of thiomethyltetrazine probes were locked in live cells to show different labeling profiles by western blot analysis. The three probes were further evaluated to identify unique pathways in a live-cell proteomic study. We anticipate that the trifold function of thiomethyltetrazines as electrophilic warheads, bioorthogonal reporters, and switches for "locking in" stability will facilitate future discovery efforts.

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ACKNOWLEDGMENT

We thank Dr. Jaimeen Majmudar (Pfizer) for insightful discussions, Dr. Andrew Jemas (UD) for his assitance with confocal imaging, Dr. Uthpala Seneviratne (AstraZeneca) for insightful proteomic discussions, and we thank Dr. Yanbao Yu (UD) for assistance with proteomics. This work was supported by NIH (R01GM132460) and Pfizer. Instrumentation was supported by NIH awards P20GM104316, P20GM103446, S10OD025185, S10OD026951, S10OD016267, S10 OD016361, and S10 OD30321. Facilities and instrumentation were also supported by NSF through the University of Delaware Materials Research Science and Engineering Center, DMR-2011824.

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