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Review

Decoding Emerging Protein Post-Translational Modifications Through Chemical Biology

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Abstract

Protein post-translational modifications (PTMs) greatly expand proteome complexity by regulating protein activity, localization, stability, and intermolecular interactions. While advances in mass spectrometry have enabled the large-scale characterization of canonical PTMs, the discovery and analysis of newly identified rare modifications remain technically challenging because of their limited abundance, dynamic nature, and lack of robust enrichment methods. Recent advances in chemical biology have provided powerful complementary approaches that overcome many of these limitations by exploiting the chemical reactivity, metabolic origin, or enzymatic installation of PTMs. In this review, we summarize emerging chemical biology strategies that have accelerated the profiling and functional characterization of novel protein PTMs and the proteoforms they generate. We discuss metabolic labeling using bioorthogonal metabolite mimics, chemoselective derivatization of endogenous PTMs, cleavable enrichment strategies for site-specific PTM identification, proximity labeling approaches for discovering PTM-associated protein interactions, and methods for site-specific PTM installation through genetic code expansion and chemical ligation. We further highlight representative applications demonstrating how these technologies have enabled the discovery of previously unrecognized PTMs, improved proteome-wide mapping, identified PTM readers, writers, and erasers, and facilitated mechanistic studies in diverse biological and disease contexts. Finally, we discuss the strengths and limitations of current methodologies and outline future directions integrating chemical biology with quantitative proteomics, single-cell analyses, and artificial intelligence-driven data interpretation. Together, these complementary technologies are reshaping the study of protein PTMs and will continue to advance our understanding of proteome regulation in health and disease.

Keywords: post-translational modifications; chemical biology; bioorthogonal chemistry; chemical proteomics; cellular proteome; metabolism

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Academic Editor: Masaru Miyagi

Received: 6 August 2026
Revised: 14 October 2026
Accepted: 26 October 2026
Published: 28 October 2026

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1. Introduction

The canonical protein biosynthesis pathway is fundamentally constrained to twenty amino acids, which imposes an inherent ceiling on the chemical diversity of the resulting proteome. Post-translational modifications (PTMs) partially overcome this limitation by expanding the chemical repertoire of the proteome after translation by generating distinct proteoforms from a single gene product, either through enzymatic catalysis or spontaneous non-enzymatic reactions (Figure 1) [1–8]. While many PTMs such as phosphorylation,

acetylation, and ubiquitylation have been extensively characterized, a growing number of chemically and functionally distinct protein modifications continue to be discovered, with roles in transcriptional regulation, signal transduction, and disease pathogenesis [9–12]. These emerging protein PTMs are typically low in stoichiometry and abundance relative to their well-characterized counterparts, necessitating advanced enrichment and detection strategies for their systematic characterization [13–16].

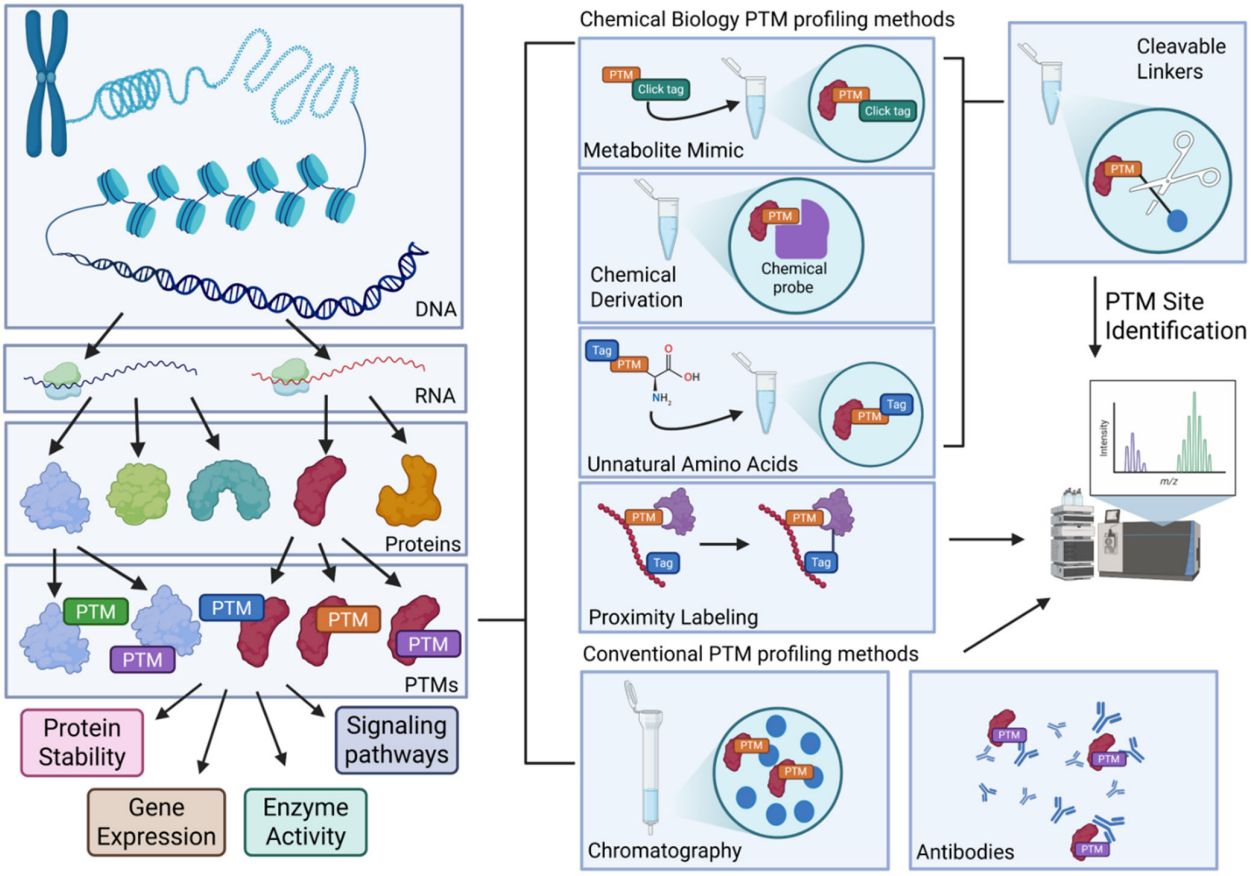


Figure 1. A summary of PTM roles in expanding the diversity and functionality of the cellular proteome and the major methodologies for profiling novel protein PTMs.

Enrichment prior to mass spectrometric analysis is a requirement for PTM profiling, even for abundant, well-studied modifications. Historically, two varieties of methods have dominated this effort: affinity chromatography exploiting the physicochemical changes specific PTMs induce on proteins, and immunoprecipitation using modification-specific antibodies [17–21]. Chromatographic methods are highly effective for chemically distinctive modifications; however, this method is inherently limited to PTMs that sufficiently affect the physicochemical properties of the proteins they modify to permit selective capture. Antibody-based enrichment is more broadly applicable to PTM selective capture but carries well-documented analytical liabilities including batch-to-batch variability, context-dependent epitope recognition, and significant rates of off-target binding [22,23].

Alternatively, chemical biology approaches, specifically bioorthogonal chemistry-based strategies, offer solutions to many of these limitations (Figure 1). Due to recent advances in this field, there has been an explosion of complementary techniques to study PTM proteoforms that utilize motif-specific reactions, enzyme engineering, metabolite mimics, as well as many other chemical biology methodologies [24–31]. Rather than relying on non-covalent recognition by antibodies, many of these methods directly exploit the chemical reactivity, metabolic origin, or enzymatic installation of a PTM. Such strategies can enable

temporal control [32,33], improved molecular definition [34,35], and selective enrichment of PTM-containing proteins or peptides [36,37]. Importantly, chemical biology tools are not intended to replace conventional enrichment methods universally; instead, they provide orthogonal capabilities that are particularly valuable for low-abundance, dynamic, or poorly characterized PTMs.

In parallel with these wet-lab advances, computational and artificial intelligence (AI)-driven methods have become increasingly central to PTM discovery and validation. Deep learning-based de novo peptide sequencing and open-search algorithms have improved the ability to identify unanticipated mass shifts corresponding to novel or low-abundance modifications, reducing reliance on restrictive, modification-specific database searches [38,39]. Machine learning models trained on large-scale PTM datasets have also enabled prediction of modification sites, functional consequences, and enzyme-substrate relationships, helping prioritize candidates for targeted chemical biology validation [40–42]. Rather than functioning as a standalone solution, AI-assisted analysis is best understood as complementary to enrichment chemistry. Computational tools can flag candidate modifications and modification sites on proteins at scale, while chemical biology strategies provide the selective, mechanistically grounded validation needed to confirm their biological relevance.

In this review, we delve into the novel chemical biology methods that have been employed to profile and investigate emerging protein PTMs, situating these strategies alongside the complementary analytical and computational advances that together are expanding the frontier of discovery in the field of PTM proteoforms.

2. Classical Proteomics Methods for PTM Studies

The foundational workflow for PTM proteomics couples an enrichment step (either chromatographic or immunoaffinity) with a mass spectrometric acquisition strategy that best fits the experimental question. Prior to the widespread adoption of the chemical biology tools described in subsequent sections, these enrichment methods constituted the primary means by which PTMs could be systematically profiled at the proteome scale. As mentioned in the introduction, chromatographic enrichment is effective for PTMs that confer a sufficiently distinct physicochemical adjustment to the modified peptide. Phosphopeptides are selectively captured by IMAC or TiO_2 chromatography through the affinity of the phosphate moiety for metal ions, while glycopeptides can be enriched by lectin affinity chromatography or hydrophilic interaction liquid chromatography (HILIC) based on their hydrophilicity and carbohydrate-recognition interactions [43–46]. However, these approaches are inherently PTM-specific and lack generalizability to the modified proteome because they require either a known high-affinity capture interaction or a physicochemical property that sufficiently differentiates modified from unmodified proteins or peptides.

Immunoaffinity enrichment using modification-specific antibodies offers an alternative with broader PTM coverage when compared to chromatography and has been the dominant approach for profiling marks such as lysine acetylation, methylation, and ubiquitylation at the proteome scale [47–49]. The practical versatility of antibodies, such as their applicability across western blotting, immunoprecipitation, flow cytometry, and enrichment workflows, has contributed to their widespread adoption for the study of proteins modified by PTMs [23,50,51]. Nevertheless, antibody-based approaches carry well-documented analytical limitations that are intrinsic to the methodology itself. A systematic evaluation of commercial antibodies found that approximately 25% failed specificity validation and 20% were non-functional in chromatin immunoprecipitation assays [52]. At the molecular level, antibody recognition depends on noncovalent binding to the modified epitope in the context of the flanking protein sequence, which means specificity is inherently susceptible to steric and electrostatic effects from neighboring amino acid residues, introducing systematic

biases in site identification. Additional limitations include batch-to-batch variability in antibody performance, absence of temporal resolution, time-consuming modification-specific antibody development, and the inability to distinguish between the activities of distinct writer enzymes that install the same chemical modification at different sites [22,23,52].

It is also essential to mention gel-based platforms, most notably high-resolution 2D gel electrophoresis (2DE), that separate intact proteoforms directly utilizing isoelectric point and molecular weight, completely bypassing the need for PTM-specific capture reagents. When coupled to intact-mass identification, 2DE-based workflows can determine PTM identity without relying on a known affinity interaction and continue to provide the highest achievable resolution of proteoforms across the full breadth of a proteome [53]. In fact, recent quantitative assessments of optimized 2DE/LC-MS/MS top-down proteomic workflows have confirmed that this coupling represents the most efficient approach to broadly and deeply resolve proteomes at the level of their constituent proteoforms [54]. PTM-specific in-gel fluorescent stains further allow 2DE to be applied selectively to modified subproteomes (such as stains specific to phosphoprotein and glycoprotein) without requiring mass spectrometry for initial detection [55].

Recent methodological refinements have directly addressed several of the limitations historically associated with 2DE. Optimized sample reduction protocols substantially increase resolvable spot counts and reduce streaking, gel-based prefractionation strategies such as PEPPI-MS extend intact proteoform and protein-complex analysis to species otherwise lost to complexity, dynamic-range compression via combinatorial peptide ligand libraries unmasks low-abundance species prior to electrophoresis, and automation of downstream identification via high-throughput MALDI-TOF/IEF workflows has begun to narrow the throughput gap with LC-MS/MS-based approaches [56–60]. Regardless of these advances, a few limitations for gel electrophoresis remain. Gel electrophoresis is low throughput compared to many of the other techniques mentioned in this review. Due to the separation method used by gel electrophoresis, the technique also performs poorly for proteins that are very large, very small, highly hydrophobic, or extreme in isoelectric point. It is also important to mention that if the goal is isolating the proteome based on a specific PTM rather than individual proteoforms, gel electrophoresis is not the most optimized technique, as it distinguishes between modified proteins and unmodified proteins but does not isolate proteins based on PTM.

Quantitative proteomic strategies including stable isotope labeling by amino acids in cell culture (SILAC) and isobaric tandem mass tag (TMT) labeling do not function as enrichment tools but instead enable comparative abundance profiling of PTM proteoforms across experimental conditions. These approaches have been applied to characterize PTM dynamics in response to pharmacological perturbation, during cell signaling cascades, under conditions of controlled protein degradation, and across different cellular growth states [61–64]. Contemporary advances in quantitative proteomics have focused on integrating these labeling strategies with novel enrichment workflows and on the development of improved reagents to increase multiplexing capacity, quantitative accuracy, and compatibility with low-input samples [65–69].

3. Chemical Biology Tools and Approaches for PTM Studies

The following sections discuss the most representative chemical biology strategies for PTM profiling, organized by methodological approach. Each strategy is examined with respect to its chemical logic, representative applications in the PTM field, and inherent limitations.

3.1. Metabolic Labeling of Target PTMs Using Metabolite Mimic Probes

The majority of PTMs are installed by enzymes or are nonenzymatically added to proteins and are based on endogenous small-molecule substrates such as acyl-CoA thioesters, SAM, nucleotide-sugars, or amino acid derivatives whose cellular ubiquity makes the selective targeting of the resulting modifications analytically challenging. Even the molecules used for novel PTMs such as monoaminylation originate from endogenous processes or diet. Bioorthogonal metabolite mimics address the selectivity problem by introducing a structural analog of the endogenous substrate. This mimic contains a reactive functional group, most commonly an azide or terminal alkyne, that is absent from biological environments and therefore amenable to selective covalent capture via bioorthogonal click chemistry reactions. The key design requirement is that the structural perturbation introduced by the clickable handle does not substantially impair the ability of the relevant enzymes to utilize the analog as a substrate for modifying the proteome (Figure 2) [70,71].

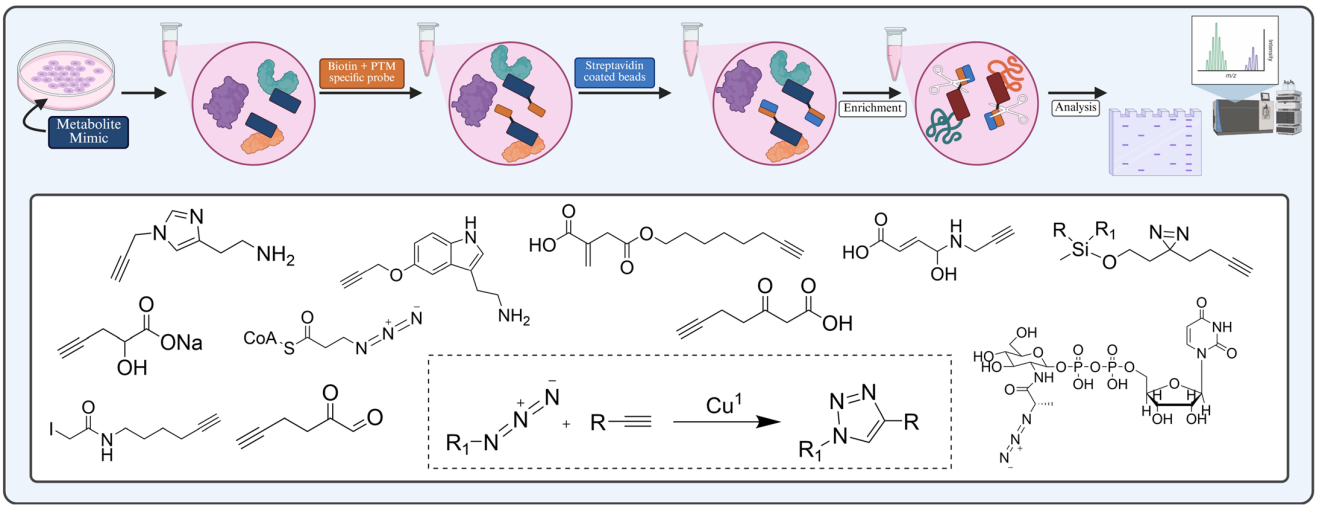


Figure 2. A workflow of metabolic labeling of target PTMs using metabolite mimic probes and the structures of representative probes.

Following the foundational development of bioorthogonal chemistry by Bertozzi in the early 2000's, metabolic labeling workflows were rapidly adapted for PTM proteomics by multiple groups [72–77]. These papers lead to the eventual converging of the field to a common operational paradigm: the analog is administered to cultured cells or organisms, time is allowed for its enzymatic incorporation into the target PTM, cells are lysed under denaturing conditions, and modified proteins or peptides are covalently captured via a click reaction (such as copper-catalyzed azide-alkyne cycloaddition (CuAAC) or strain-promoted [3 + 2] cycloaddition (SPAAC)) using a biotinylated click reagent to enable streptavidin-based affinity enrichment prior to mass spectrometric identification [35,78,79]. This approach has been applied to profile numerous well-established PTMs including O-GlcNAcylation, S-palmitoylation, and lactylation and has more recently enabled the characterization of emerging modifications [80–82].

Lactylation, the acylation of lysine residues by lactyl groups derived from lactate, represents a compelling example of how metabolic labeling can drive the functional characterization of a novel PTM. First identified as a histone modification capable of activating gene expression, lactylation is particularly relevant in oncology given the Warburg effect-driven elevation of intracellular lactate in tumor cells [83,84]. Introduction of an alkyne-functionalized lactate analog into cells enabled proteome-wide mapping of lactylation sites, identifying 114 modified sites across 62 proteins. Among these, PARP1 was subsequently

validated as a physiologically relevant lactylation substrate, with the lactylated PARP1 proteoform shown to potentiate ADP-ribosylation activity and implicate lactylation in the regulation of DNA repair [78].

Metabolic labeling has also been instrumental in characterizing the biologically important class of histone monoaminylations, which is the transglutaminase-mediated conjugation of biogenic amines including serotonin, dopamine, and histamine to glutamine residues on histone tails [85–88]. Using bioorthogonal amine analogs in combination with chemoproteomics, the transglutaminase 2 enzyme was identified to be more than just a writer of histone H3 monoaminylation but also to function as an eraser and exchanger of these modifications. These modifications (specifically histaminylation and serotonylation) were further shown to regulate the expression of circadian rhythm genes [89].

The utility of metabolic labeling is further enhanced by its compatibility with different chemical biology methodologies. Enzyme engineering strategies can expand the substrate tolerance of biosynthetic enzymes toward structurally bulky bioorthogonal analogs that would not be utilized by the endogenous version of the enzyme to modify the proteome. For instance, structure-guided mutagenesis of GalNAc-transferases enabled incorporation of a sterically demanding GlcNAc analog that would be rejected by wild-type enzymes, allowing selective proteome-wide mapping of O-GalNAc glycosylation substrates for individual transferase proteoforms [28]. This study utilizes an additional chemical biology methodology to improve probe bioavailability and cellular selectivity. This methodology is the use of pro-drug-like caging strategies, in which reactive or membrane-impermeant functional groups on the analog are masked with protecting groups that are removed intracellularly by enzymatic activity, pH-dependent hydrolysis, or photochemical deprotection [90–93]. Specific to the aforementioned GalNAc study, S-acyl-2-thioethyl (SATE) protecting groups on the phosphate oxygens of the nucleotide-sugar analog were utilized to render the probe cell-permeable while being cleaved intracellularly to release the active substrate.

An analogous bump-and-hole strategy has been applied to acyl-CoA-dependent writers. A panel of acetyl-CoA derivatives bearing azide or alkyne handles was paired with active-site-enlarged lysine acetyltransferase (KAT) variants, yielding bioorthogonal enzyme–cofactor pairs capable of labeling substrates in the presence of the competing endogenous acetyl-CoA pool [94]. Applied with 3-azidopropanoyl-CoA, this approach assigned over four hundred candidate substrates to GCN5 and p300 and subsequently validated IQGAP1 and SMC1 as previously unrecognized GCN5 targets [95]. Because 3-azidopropanoyl-CoA proved cell-permeable, the strategy was subsequently extended into living cells: an engineered histone acetyltransferase (HAT1) variant combined with SILAC proteomics resolved 123 HAT1-specific substrates in their native cellular context [96]. The same logic has since been transferred to the protein S-acyltransferases, where a "hole" ZDHHC mutant paired with a "bump" lipid probe identified more than 300 isoform-specific S-acylation substrates [97]. Writer-resolved profiling of this kind directly addresses a limitation of antibody-based enrichment noted in Section 2, namely the inability to distinguish the activities of distinct writer enzymes installing the same chemical mark.

This metabolite mimic method solves many of the fundamental issues that are inherent to the well-established methods mentioned in Section 2. The method has the potential for temporal resolution through pulse-chase experiments; the minimal click handle reduces steric effects significantly; there is no variability between probe batches due to a defined chemical structure; it can be coupled with quantitative methods such as TMT; and these mimics can be engineered to have writer specificity [96–98]. Regardless of these strengths, metabolic labeling is subject to inherent limitations that stem from the structural inconsistencies between the analog and the endogenous metabolite. The introduction of even a minimally perturbing clickable handle alters the steric and electronic properties of the sub-

strate, which can reduce enzymatic processing efficiency, distort substrate selectivity, alter the in vivo metabolic fate of the analog, or introduce cytotoxic effects at the concentrations required for adequate labeling efficiency. These factors allow the potential for false-positive signals through incorporation of the analog by off-target enzymes and false-negative results because there is an inevitable incomplete competition between the endogenous metabolite and the mimic. It is also important to mention that the area around the modification site can impact the level at which the click reaction can occur. If an area is less accessible, it is possible the protein will not be identified. Rigorous validation of any metabolic labeling probe therefore requires biochemical confirmation of on-target enzymatic incorporation, demonstration of competitive displacement by the endogenous substrate, and validation of representative identified sites (Figure 2) [27–29,33,65,99].

3.2. Chemical Derivatization of Endogenous PTMs Using Bioorthogonal Probes

Chemoselective derivatization offers a complementary strategy for profiling PTM-modified proteins that circumvents the requirement for exogenous metabolic mimic introduction into living cells. Rather than relying on biosynthetic incorporation of an analog, these methods exploit the intrinsic chemical reactivity of the PTM itself to install a detectable or enrichable tag directly onto the endogenous modification. This approach preserves the modification's native chemistry and avoids the potential for biological perturbation inherent to metabolic labeling. However, because reactions must be performed under conditions compatible with protein integrity while also being highly selective for a given modification, they frequently require non-physiological conditions (metal catalysts, extreme pH, nucleophilic reagents) that cannot always be applied to intact cells (Figure 3).

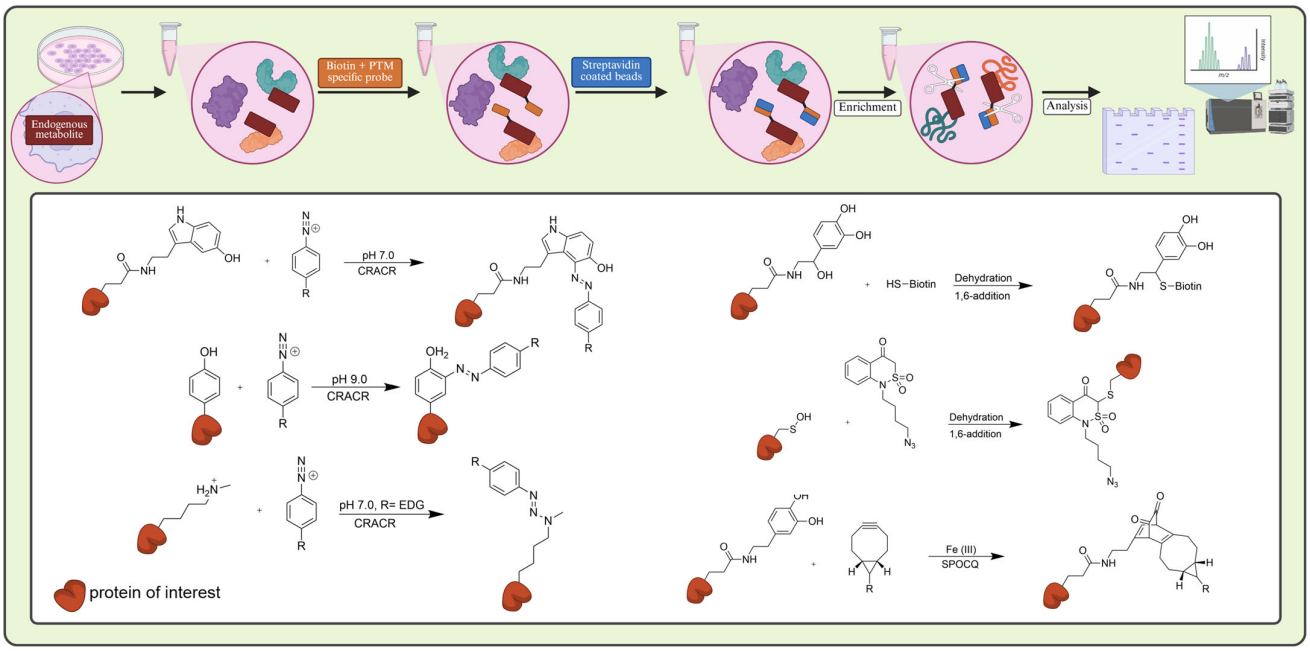


Figure 3. A workflow of chemical derivatization of endogenous PTMs using bioorthogonal probes and the representative chemoselective bioconjugation reactions.

Selectivity is the central analytical challenge in chemoselective derivatization. Highly abundant functional groups such as amines and thiols are present throughout the proteome, necessitating either highly specific chemical reagents or protection-deprotection strategies to distinguish the PTM of interest from background reactivity. A well-characterized example of intrinsic chemical selectivity is provided by the chemoselective rapid azo-coupling reaction (CRACR) for serotonylation profiling [100,101]. Aromatic diazonium ions undergo

electrophilic coupling with electron-rich aromatic systems in a pH-dependent manner: at pH 9, coupling is dominated by tyrosine residues, while at pH 6–7 selectivity shifts toward 5-hydroxyindole-containing substrates, including the serotonin moiety in glutamine serotonylation. This differential reactivity was exploited to selectively label serotonylated proteins in cell lysates and profile the serotonylated proteome of colorectal cancer cells [101]. The chemical scope of diazonium-based derivatization has subsequently been extended to monomethyl lysine, through the incorporation of electron-donating substituents on the arene diazonium scaffold that increase its electrophilicity toward the weakly nucleophilic secondary amine of the methylated lysine side chain [102]. Similarly, the strain-promoted oxidation-controlled cyclooctyne-1,2-quinone cycloaddition (SPOCQ) enables chemoselective labeling of dopaminylated proteins by reacting with the ortho-quinone form of dopamine produced through the oxidation of the catechol group [86,103].

For PTMs involving more common functional groups, a protection-deprotection strategy is typically required to achieve selectivity. The acyl-biotin exchange (ABE) method for S-palmitoylation profiling illustrates this principle. Free thiols are first capped with N-ethylmaleimide to prevent non-specific reactions. The thioester bond of the palmitoyl modification is then selectively cleaved by hydroxylamine, liberating the formerly acylated cysteine thiol. The unmasked thiol is subsequently conjugated with a biotinylated thiol-reactive reagent for affinity capture [104]. A quantitative variant of this approach replaced the biotin tag with an iodoacetamide-based acylation site-identifying reagent to enable gel-based quantification of palmitoylation levels [105]. Analogous protection-deprotection or selective derivatization strategies have been developed for glycosylation, acetylation, citrullination, and S-nitrosylation [106–109].

Despite these advantages, chemoselective derivatization remains fundamentally constrained by reaction selectivity as previously mentioned. Many biological functional groups are chemically similar and occur at high abundance throughout the proteome, leading to an abundance of off-target reactions. Apparent selectivity may also depend strongly on buffer composition, pH, reagent concentration, reaction time, and sample preparation conditions. The reactions must be mild enough to preserve the native protein structure, which caps the achievable conversion rate, leading to incomplete reaction with the endogenous metabolite. Therefore, rigorous controls are essential, including no-reagent controls, omission of key activation or cleavage steps, competition experiments where applicable, validation by targeted mass spectrometry or biochemical assays, and many experimental repeats. In cases where wet lab controls are not capable of sufficiently eliminating bias, rigorous computational controls are important for establishing confidence in the proteomic data. Rather than serving as a universal replacement for antibody-based or metabolic-labeling workflows, chemoselective derivatization is similar to chromatographic methods that are most powerful when applied to PTMs with distinctive and experimentally accessible chemical reactivity (Figure 3) [86,100–109].

3.3. Cleavable Linkers for PTM Site Analysis

A critical but often underappreciated component of PTM proteomics workflow performance is the molecular architecture of the bifunctionalized enrichment reagent that connects the clickable handle on the metabolic probe or derivatization tag to the streptavidin-functionalized molecule used for affinity capture. In its simplest form, this reagent presents a bioorthogonal functional group complementary to the click handle on the PTM probe at one end and a biotin group at the other, enabling streptavidin-based pulldown of probe-labeled proteins to be subsequently digested with trypsin and analyzed by LC-MS/MS. While effective for PTM protein-level identification, this architecture presents a fundamental limitation for PTM site localization: after on-bead trypsin digestion, the modified peptide bearing the

enrichment reagent remains covalently tethered to the bead through the biotin-streptavidin interaction, preventing its elution and mass spectrometric sequencing. Incorporation of a chemically cleavable linker between the bioorthogonal handle and the biotin group resolves this problem by permitting selective release of modified peptides from the solid support under defined conditions, enabling direct identification of the modified residue by MS/MS fragmentation (Figure 4) [110,111].

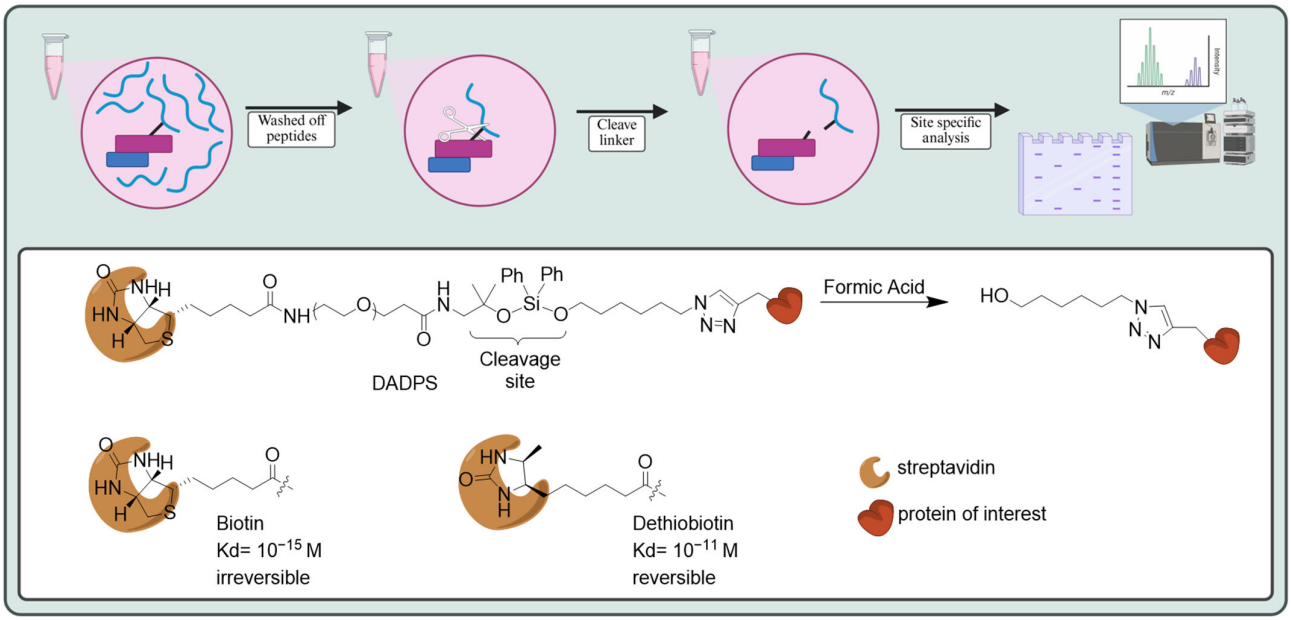


Figure 4. A workflow of using cleavable linkers for PTM site analysis and the representative linker structures.

Several cleavable linker chemistries have been developed for the application of PTM site identification, each with distinct practical trade-offs. The dialkoxydiphenylsilane (DADPS) reagent has emerged as the gold standard, incorporating a phenylsilane moiety that undergoes hydrolytic cleavage under mildly acidic aqueous conditions (e.g., 10% formic acid), which are fully compatible with standard reversed-phase LC-MS/MS sample preparation [31]. A systematic comparison of the major cleavable enrichment architectures such as photocleavable reagents (Az-UV-biotin and Az-PC-biotin, cleaved by UV irradiation); hydrazine-cleavable reagents (Az-Dde-Biotin); reductively cleavable reagents (Az-Diazobiotin, cleaved by phosphine reduction); and the aforementioned DADPS demonstrated that DADPS achieved the highest PTM site profiling efficiency, covering up to 96% of the total modified peptides found with all linkers under optimized conditions [112]. An alternative strategy that avoids the need for a covalent cleavage reaction entirely employs desthiobiotin in place of biotin as the streptavidin-binding moiety. Desthiobiotin binds streptavidin with significantly lower affinity than biotin (Kd ~10^{-11} M versus ~10^{-15} M), enabling competitive displacement of desthiobiotinylated peptides by free biotin in a simple elution step without requiring a chemical cleavage reaction [113].

Each cleavable enrichment strategy carries specific technical limitations that must be considered in the context of the target PTM and downstream analytical workflow. Photocleavable reagents and desthiobiotin-based elution are both susceptible to incomplete release, resulting in reduced recovery of modified peptides. Desthiobiotin enrichment is further complicated by competition from endogenous biotinylated proteins and free biotin present in cell lysates. This biotin can partially saturate streptavidin binding sites and prevent the binding of desthiobiotinylated probes prior to the elution step, a more significant problem than for conventional biotin-based capture given the weaker binding affinity of

desthiobiotin. The acidic hydrolysis conditions used to cleave the phenylsilane in DADPS, while mild relative to many chemical cleavage reactions, are incompatible with acid-labile PTMs such as O-glycans, restricting its applicability in glycoproteomics workflows. Reductively cleavable reagents require high concentrations of reducing agent (typically TCEP or sodium phosphine) and high-salt buffer conditions, necessitating an additional desalting step before LC-MS/MS injection to prevent ion suppression. A universal analytical consideration applicable to all cleavable linker strategies is that cleavage leaves a residual mass adduct (the stub of the linker remaining on the modified residue) which must be accounted for in database search parameters. Further, this mass adduct may alter peptide fragmentation behavior, thereby affecting sequence coverage or complicating spectral interpretation [110–113]. Notwithstanding these limitations, cleavable enrichment handle strategies represent the current methodological standard for PTM site-level identification by chemoproteomics, as no alternative approach provides equivalent capacity for direct, broadly applicable localization of modification sites on enriched peptides at proteome scale (Figure 4).

3.4. Proximity Labeling Approaches for PTM Reader Identification

A methodologically distinct class of chemical biology tools for PTM research exploits proximity-dependent enzymatic biotinylation to map the protein interaction landscape associated with specific PTMs by proteins that interact with said PTMs. Proximity labeling enzymes such as the biotin ligase variants BioID, BioID2, TurboID, and BASU, as well as the ascorbate peroxidase APEX2, catalyze the generation of short-lived, highly reactive biotin intermediates (biotinoyl-AMP or biotin-phenoxyl radicals, respectively) that covalently label proximal proteins within a radius of approximately 10 nm [114–116]. By genetically or biochemically targeting these enzymes to a specific PTM site, it is possible to capture transient and low-affinity interactions between a modification and its reader, writer, or eraser proteins that are lost when the cell is lysed under standard conditions (Figure 5).

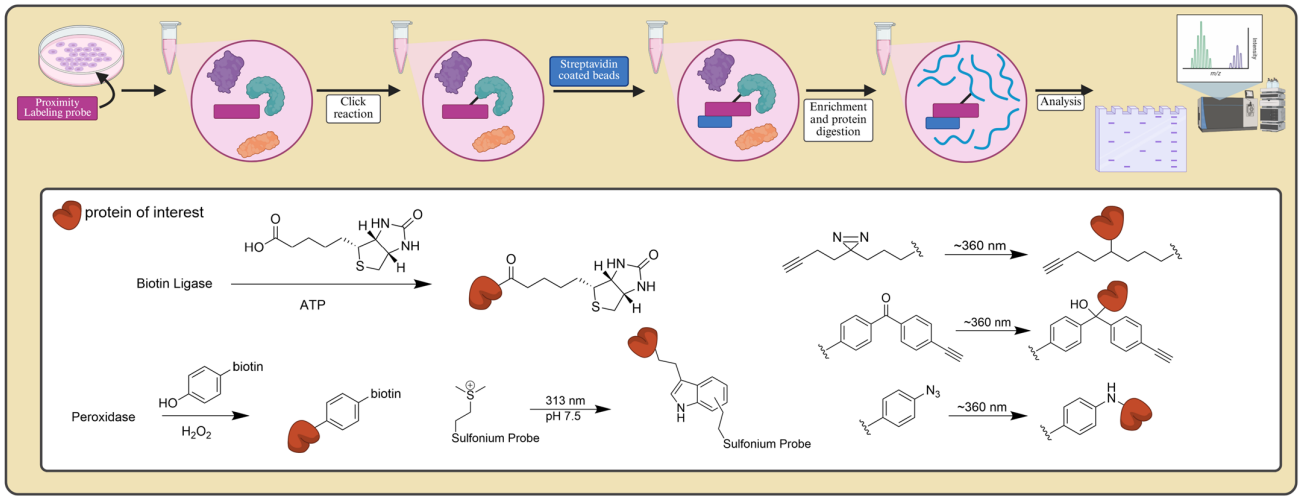


Figure 5. A workflow of proximity labeling approaches for PTM reader identification and the representative crosslinking reactions.

The most conceptually direct application of proximity labeling to PTM biology is the identification of reader proteins for a given PTM. The ChromID platform exemplifies this approach: modification-selective chromatin reader domains are fused to biotin ligases (BioID2 or BASU) and expressed in cells, resulting in biotinylation of the local proteome surrounding specific histone marks [117]. Applied to H3K4me3, ChromID identified established transcription factors, chromatin remodeling complexes, and histone acetyltrans-

ferases at this mark, as well as previously uncharacterized candidate reader proteins. An antibody-based variant, AMAPEX (antibody-mediated protein A-ascorbate peroxidase 2 labeling), substitutes an antibody for the engineered reader domain, enabling proximity labeling at endogenous modification sites without requiring a characterized reader protein. Comparative analysis demonstrated that AMAPEX achieved significantly improved nuclear localization of labeled proteins (70% nuclear) relative to conventional antibody-HRP approaches (40% nuclear), reflecting the superior spatial resolution of APEX2-based labeling [118]. More recently, Chroma-μMap combined photocatalytic proximity labeling with modification-specific antibodies to achieve a sub-nanometer labeling radius, enabling discrimination of reader complexes at spatially adjacent histone proteoforms [119].

A complementary, non-enzymatic strategy for reader identification employs synthetic PTM-bearing peptides as photoaffinity baits rather than relying on genetically encoded proximity labeling enzymes. In this approach, a histone (or other substrate) peptide is synthesized bearing the PTM of interest alongside two additional functionalities: a photoreactive crosslinking group, most commonly benzophenone or a diazirine, and a terminal alkyne handle or biotin molecule for downstream bioorthogonal conjugation. Upon incubation with cell lysate or nuclear extract and subsequent UV irradiation, the photoreactive group converts transient, non-covalent reader-PTM interactions into a stable covalent product, after which the alkyne handle enables click-based conjugation of a fluorophore for visualization or a biotin tag for affinity enrichment and mass spectrometric identification [120,121]. This strategy was first demonstrated using benzophenone-based photoaffinity probes to capture readers of trimethylated histone H3 lysine 4 (H3K4me3), and diazirine-based probes were subsequently developed as a higher-yielding, more specific alternative, achieving improved photocrosslinking efficiency relative to the benzophenone scaffold [120]. Beyond reader identification, this same probe architecture has been extended to capture PTM erasers, including enzymes that remove histone lysine acetylation and malonylation, by trapping the transient enzyme-substrate interaction that forms prior to catalytic turnover [121].

Proximity labeling approaches carry inherent limitations in PTM proteoform interaction contexts. The first fundamental design problem in proximity labeling approaches is the fact that some level of structural knowledge is required when designing proximity labeling molecules. Once this design is made, the primary challenge is achieving specific localization of the labeling enzyme to the modified rather than unmodified form of the target protein, particularly for modifications of low stoichiometry. This challenge branches out even further into the issue of false positives, as it is difficult to discern if an interacting protein is modification-specific. Antibody-based targeting strategies address this partially but introduce the selectivity limitations of antibodies discussed in previous sections. Additionally, because enzymatic proximity labeling captures all proteins within the labeling radius over a finite exposure window regardless of their relationship to the PTM of interest, the resulting datasets necessarily include background signal from abundant proximal proteins not functionally associated with the modification proteome while also being biased towards proteins that interact for a longer amount of time. Careful experimental design including competitive antibody blocking controls, comparison with unmodified locus controls, and integration with complementary interaction data is essential to distinguish modification-specific interactions from background biotinylation (Figure 5) [114–121].

3.5. Delivery of the Target PTMs into the Cellular Proteome

A conceptually distinct approach to PTM functional characterization employs genetic code expansion (GCE) or chemical ligation strategies to install a defined modification or a close structural mimic at a specific site within a target protein. Unlike the discovery-oriented methods described above, these approaches do not identify novel PTM sites but

instead enable rigorous biochemical and cellular characterization of known modifications by providing access to homogeneously modified proteoforms in quantities sufficient for structural, enzymatic, and cell biological studies (Figure 6).

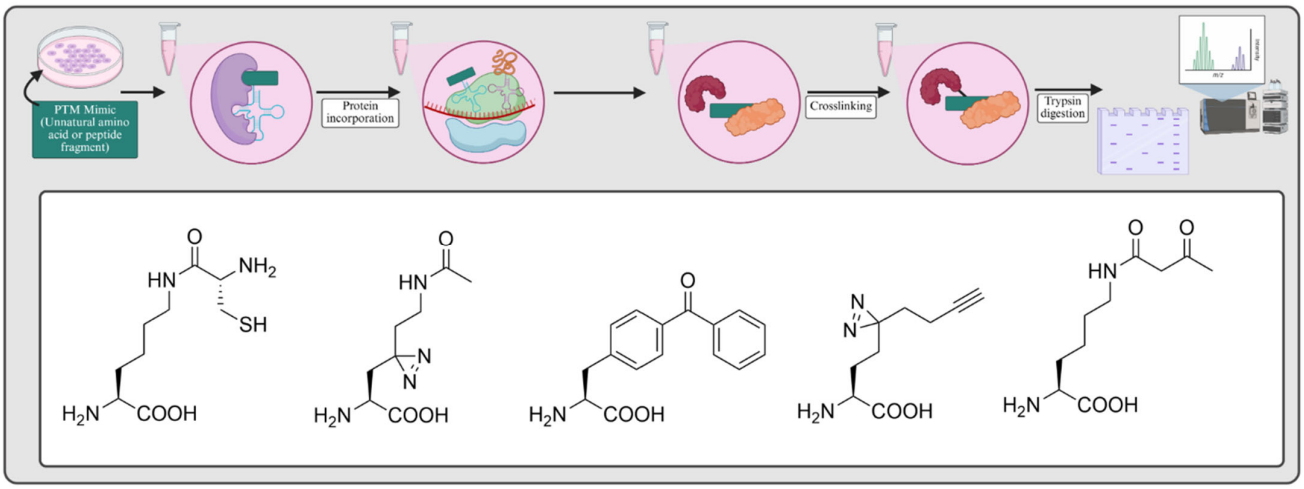


Figure 6. A workflow for delivering the target PTMs into the cellular proteome using genetic code expansion and the representative unnatural amino acids used for PTM studies.

Genetic code expansion leverages the engineering of orthogonal aminoacyl-tRNA synthetase (aaRS)/tRNA pairs, most commonly derived from the pyrrolysyl system of methanogenic archaea, to enable the ribosomal incorporation of non-canonical amino acids (ncAAs) in response to an amber (UAG) stop codon introduced by site-directed mutagenesis. The chemical scope of GCE has been substantially expanded through rational engineering of the pyrrolysyl-tRNA synthetase (PylRS) active site with one systematic approach using structure-guided mutagenesis, achieving up to six-fold improvements in ncAA incorporation efficiency for specific substrates [122]. GCE has been applied to install PTM mimics including acetyl-lysine, propionyl-lysine, and various acylation marks, as well as being combined with metabolic labeling and chemoselective derivatization strategies [123–126]. A recent study in the Huang laboratory illustrates this combinatorial potential by utilizing a chemoselective probe for lysine acetoacetylation integrated into the proteome with GCE to simultaneously profile the acetoacetylation landscape proteome-wide and validate the functional consequences of site-specific modification (Figure 6) [127].

Chemical ligation encompasses two principal strategies. Native chemical ligation (NCL) exploits the chemoselective reaction between a C-terminal thioester and an N-terminal cysteine to join two unprotected peptide or protein fragments under aqueous conditions, enabling the synthesis of specific proteoforms bearing defined modifications within the ligation region. Expressed protein ligation (EPL) extends this approach by producing one fragment as a recombinant intein fusion protein, allowing access to larger modified proteins than is feasible by total synthesis alone. The utility of NCL for PTM functional studies was demonstrated in early work on chromatin biology, including the synthesis of histone H4 site-specifically acetylated at K16 to investigate the role of this modification in nucleosome compaction [128]. While chemical ligation has been predominantly applied to the preparation of modified proteins for in vitro biochemical and structural studies, recent work has demonstrated that split-intein systems can mediate post-translational modification of histone tails directly in cells, establishing a proof-of-concept for intracellular chemical ligation as a strategy for studying PTM function on proteins in a cellular context [129].

Enzymatic ligation offers a complementary route to intracellular PTM installation. Sortase A, a bacterial transpeptidase natively involved in peptidoglycan biosynthesis, recognizes

a C-terminal LPXTG sorting motif and forms an acyl-enzyme thioester intermediate that is subsequently resolved by an N-terminal glycine-glycine nucleophile on a second substrate, a reaction long exploited in vitro as sortase-mediated ligation (SML) for producing site-specifically modified proteins. This strategy was extended by developing sortase-mediated metathesis (SMM), which exploits the same transpeptidation equilibrium to exchange an existing C-terminal tail for a synthetic, PTM-bearing peptide without requiring the substrate to first be engineered with the canonical N- or C-terminal sorting sequences. Using an engineered, calcium-independent sortase variant (6M-Srt) generated by combining activating mutations identified through structure-guided mutagenesis, a synthetic histone H3 N-terminal peptide bearing defined PTMs was delivered into live cells and metathesized directly onto endogenous, chromatin-bound histone H3 in place of its native N-tail [130–132]. This represents the first demonstration that an enzymatic ligation strategy can edit native histone PTM patterns on chromatin in cellulo, in contrast to genetic code expansion and protein trans-splicing approaches, which are generally limited to editing newly synthesized histone prior to chromatin deposition and cannot access the pre-existing, chromatin-incorporated histone pool [133].

Both GCE and chemical ligation are subject to technical constraints that define their appropriate application domains. GCE is limited to the incorporation of a single ncAA per open reading frame using the amber codon suppression strategy (a consequence of the triplet codon system) and competes with termination at UAG codons, reducing incorporation efficiency. The method is further restricted to modifications that can be structurally encoded as a single amino acid residue, excluding complex multi-residue or multi-step modifications. Chemical ligation via NCL requires the presence of a cysteine residue at the ligation junction; when the native sequence lacks a cysteine at the desired site, a non-native cysteine must be introduced or generated via desulfurization of other thiol-containing residues, which adds synthetic complexity and may compromise the biochemical properties of the product. The presence of multiple cysteine residues in the protein can also reduce ligation efficiency and selectivity. Despite these constraints, GCE and chemical ligation represent irreplaceable tools for mechanistic PTM biology, providing access to defined modification states that cannot be achieved by other means [123–133].

It is worth noting that GCE and chemical ligation occupy a fundamentally different methodological niche than the profiling approaches discussed above: rather than detecting or quantifying naturally occurring PTMs within a complex proteome, these tools generate homogeneously defined, single-proteoform material by design. The completeness, mass-action, and quantification considerations that govern proteome-scale detection methods therefore do not directly apply, and the relevant trade-off instead is between site-specific structural certainty and the practical synthetic constraints described below.

Taken together, the approaches discussed in this section differ not only in target chemistry but in the fundamental type of data they are capable of producing (Table 1). Metabolic labeling and chemoselective derivatization, even when paired with isotopic or isobaric quantification, primarily support relative comparisons of PTM levels across matched experimental conditions rather than absolute stoichiometric measurement, because their signal is convolved with competing endogenous pools (metabolic labeling) or capped by the conditions required to preserve native structure (chemoselective derivatization). Cleavable enrichment handles offer the most direct route to unambiguous site-level qualitative identification but remain similarly limited to relative quantification across identically processed samples by the compounding losses inherent to multi-step workflows. Proximity labeling is, strictly speaking, orthogonal to PTM quantification altogether: it reports the relative enrichment of candidate interactors, not the occupancy of the modification itself, and is further constrained by the finite, time-integrated nature of the labeling window. GCE and

chemical ligation fall outside this framework entirely, trading proteome-scale detection for the generation of compositionally homogeneous material. A second, related theme recurs across nearly every profiling approach discussed here: each is fundamentally constrained by a form of mass-action competition between analog and endogenous metabolite, between probe and off-target nucleophile, between desthiobiotinylated peptide and endogenous biotin, or between labeling enzyme and its bait's own expression level that governs the achievable completeness of the underlying chemical or enzymatic reaction.

Table 1. A table summarizing the capabilities of each technique analyzed in this review article.

Technique	In-Cellulo Compatibility	Temporal Resolution	Quantitative Capability	Site-Level Localization	Primary Instrumentation	Representative Applications	Principal Limitation
Chromato-graphic enrichment	No	None	Relative (label-free, SILAC, TMT)	Yes, standard	LC-MS/MS	Phosphopeptides (IMAC, TiO_2); glycopeptides (lectin affinity, HILIC)	Restricted to PTMs conferring a sufficiently distinct physicochemical property
Antibody enrichment	No	None	Relative (label-free, SILAC, TMT)	Yes, standard	LC-MS/MS; WB, IP, flow cytometry	Lysine acetylation, methylation, ubiquitylation at proteome scale	Flanking-sequence epitope bias, batch variability; cannot resolve distinct writers installing the same mark
Gel electrophoresis	No	None	Relative (densitometry, intact mass)	Proteoform level, but not residue level	LC-MS/MS or MALDI-TOF/IEF; PTM-specific in-gel fluorescent stains	Broad proteoform resolution without capture reagents	Low throughput; poor for very large/small, hydrophobic, or extreme-pI proteins; does not isolate by PTM
Metabolic labeling with mimic probes (Section 3.1)	Yes	Yes (pulse-chase)	Relative; SILAC/TM T-compatible	With a cleavable linker	LC-MS/MS; in-gel fluorescence, live-cell imaging	Lactylation (PARP1), histone monoaminylation, itaconation secretome; writer-resolved: GCN5, p300, HAT1 (3AZ-CoA), GalNAc-Ts, ZDHHCs	Mass-action competition with the endogenous metabolite; the clickable handle perturbs enzyme processing; engineered-pair variants require structural knowledge
Chemoselec-tive derivatization (Section 3.2)	Limited based on probe	Limited based on probe	Relative; capped by incomplete conversion	With a cleavable linker	LC-MS/MS, 4D-DIA; fluorescence	Serotonylation (CRACR), Kme1 (STaR, >12,000 sites), dopaminylation (SPOCQ), S-palmitoylation (ABE), Cys-SOH (BTD-Az)	Selectivity against abundant competing nucleophiles; mild conditions cap achievable conversion
Cleavable enrichment linkers (Section 3.3)	Applied downstream of 3.1 and 3.2	N/A	Relative only (compounding multi-step losses); sCIP adds isobaric multiplexing	Enables site-level ID for the strategies above	LC-MS/MS; LDI-MS for the Au-nanoparticle variant	citrullination (Dde), SEE-CITE, sCIP	Residual linker mass adduct alters fragmentation and database search; DADPS acid conditions incompatible with O-glycans
Proximity/pho-toaffinity labeling (Section 3.4)	Yes (enzymatic) Lysates (photoaffinity baits)	Due to a limited labeling window, time is integrated	Reports interactor enrichment, not PTM occupancy	No	LC-MS/MS; confocal imaging	ChromID (H3K4me3), AMAPEX, Chroma-μMap, H3K4me2 sulfonium crosslinking, H3R2me2a/ H4K5acK8ac interactomes	Background from abundant proximal proteins; difficult to localize to the modified vs. unmodified proteoform
GCE/chemical & enzymatic ligation (Section 3.5)	GCE yes; NCL/EPL no; split intein and SMM yes	N/A	N/A (generates homogeneous material by design)	N/A	biochemical assays, X-ray/cryo-EM, cellular readouts	AcK/acyl-K incorporation, H4K16ac nucleosome compaction, acetoacetylation (GCE + chemoselective probe), sortase metathesis on chromatin	One ncAA per ORF (amber suppression); NCL requires Cys at the junction; not a discovery method

4. Successful Applications of Chemical Biology Tools in Protein PTM Studies

While representative examples have been incorporated into the methodological discussions above, a few recent studies warrant dedicated discussion for their technical sophistication or the biological significance of their findings, particularly those integrating multiple chemical biology strategies in a single workflow. To maintain consistency with the organizational framework of Section 3, these studies are grouped below by the primary chemical biology strategy each employs.

4.1. Applications of Metabolic Labeling

Monoaminylation, the transglutaminase-mediated conjugation of biogenic amines to glutamine residues, has emerged as an area of considerable activity in PTM biology, with recent work expanding the catalog of amine substrates and elucidating the functional consequences of site-specific modification (such as transcriptional control in the case of histone monoamine modifications). Within our laboratory, deployment of an alkyne-functionalized histamine probe in a metabolic labeling workflow identified over 450 candidate histaminylation substrates across the proteome. Among these, the histone variant H2AX was identified as a functionally significant substrate. Beyond mapping the site of modification on H2AX, we demonstrated that histaminylation modulates the formation of γH2AX, the phosphorylated proteoform of H2AX that serves as a molecular marker of DNA double-strand breaks, which has downstream consequences for cellular viability [134].

Metabolic labeling has also enabled extension of chemoproteomic profiling to PTM classes not previously accessible by this approach. An acetoacetate-alkyne probe was developed to profile crotonylation, the addition of a crotonyl group to lysine or cysteine residues, specifically in cells undergoing ketogenesis, which generates larger than average amounts of acetoacetate. Beyond identifying novel lysine crotonylation sites, this study reported the first proteome-wide evidence for cysteine crotonylation as a distinct PTM class. Functional characterization of one candidate, Cys229 of the mitochondrial peroxiredoxin PRDX3, demonstrated that the crotonylated PRDX3 proteoform impairs the enzyme's ability to detoxify reactive oxygen species, directly linking crotonylation stoichiometry to mitochondrial redox balance during ketone body metabolism [135].

The temporal dimension of PTM proteomics has similarly been addressed through metabolic labeling, in a strategy termed PTM-based secretome profiling, which combines pulse-chase metabolic labeling with protocols specifically designed to capture proteins secreted from cells over defined time windows. By coupling an itaconate-alkyne probe with secretome-selective sample preparation, this approach enables the tracking of itaconation, a non-enzymatic modification derived from the immunometabolite itaconate, on proteins as they transit through the secretory pathway, providing access to a previously inaccessible PTM-secretome axis [33].

A theme uniting several of these applications is that the metabolite mimic strategy is as readily applied to non-enzymatic modifications as to enzymatic modifications, with cysteine being the principal acceptor residue in this non-enzymatic category due to the thiolate side chain being particularly nucleophilic. The ketogenesis-derived crotonylation discussed above illustrates this principle on a single enzyme, where the crotonylated Cys229 proteoform of PRDX3 compromises reactive oxygen species detoxification [135], but the same probe logic has been applied proteome-wide to several reactive metabolites. An itaconate-alkyne probe (ITalk) that preserves the α,β-unsaturated carboxylic acid of the parent metabolite and reproduces its anti-inflammatory activity enabled quantitative, site-specific profiling of itaconation directly in living inflammatory macrophages, mapping over 1100 itaconated cysteine sites across more than 1900 proteins and being further

applied to bacterial systems [136,137]. Comparable probes have been developed for the oncometabolite fumarate, which add to cysteine thiols in a Michael-type reaction generating the fumarate-alkyne analog, S-(2-succino)cysteine. When applied in untransformed and fumarate hydratase-mutant cells, the reactivity landscape of this modification was resolved and a succination-sensitive cysteine in SMARCC1 positioned at a key protein–protein interaction interface was identified [138]. Similarly, an alkynyl analog of the lipid peroxidation product 4-hydroxy-2-nonenal permitted site-specific quantification of protein alkylation in intact cells [139]. Collectively, these studies demonstrate that metabolic labeling is not restricted to enzymatically installed PTMs but that this same bioorthogonal workflow provides proteome-wide, site-resolved access to a class of modifications that couples cellular metabolic state to protein function.

4.2. Applications of Chemoselective Derivatization

For norepinephrinylation, a distinct bioorthogonal labeling strategy was developed based on the inherent chemical reactivity of the catechol side chain of norepinephrine, circumventing the need for a pre-synthesized metabolic analog. Oxidative dehydration of the catechol to a quinonoid intermediate, followed by selective 1,6-addition of an exogenous thiol nucleophile (HS-biotin), enabled covalent tagging of norepinephrine-modified proteins directly and selectively. This approach identified over 1000 norepinephrinylated proteins with functional follow-up establishing that the norepinephrinylation of the proto-oncogenic phosphatase SHP-2 modulates its enzymatic activity, implicating this modification in oncogenic signaling [140]. Even the most well-studied monoamine modifications, such as serotonylation and dopaminylation, which are amenable to chemoselective derivatization approaches such as CRACR and SPOCQ (Section 3.2), remain incompletely understood. Work from the Maze laboratory has continued to illuminate the neurobiological roles of histone dopaminylation, identifying its impacts on synaptic plasticity and memory formation, which position this modification as a potential molecular substrate for gene regulation and the impacted protein synthesis [141].

Chemoselective derivatization of cysteine oxidative modifications has similarly enabled in vivo characterization of a transient, low-abundance PTM, the addition of sulfenic acid. BTD-Az, a cell-permeable benzothiazine-based probe bearing an azide handle, permits rapid bioorthogonal capture of cysteine sulfenic acid (Cys-SOH) both in vitro and directly in living tissue via strain-promoted azide-alkyne cycloaddition, circumventing the instability that has historically complicated sulfenylation analysis. Coupling this probe with four-dimensional data-independent acquisition (4D-DIA) proteomics enabled the profiling of more than 5000 sulfenylated proteins across mouse tissues. Applied to aging cartilage, this approach identified that the sulfenylated IDH2 proteoform promotes its own proteasomal degradation and exacerbates the redox imbalance, linking cysteine oxidation directly to age-associated mitochondrial dysfunction [142].

Methylation-specific chemoselective capture chemistry has also seen recent advances, particularly applications of the STaR strategy mentioned earlier (Section 3.2). The Wu lab developed a covalent enrichment strategy for monomethyllysine (Kme1) peptides using the aryl diazonium chemistry mentioned in Section 3.2 to address a longstanding coverage gap relative to phosphorylation and acetylation, PTMs which are routinely profiled to tens of thousands of sites. Through systematic screening, the authors identified that electron-donating dimethoxy-substituted diazonium reagents have a particularly high reactivity towards the Kme1 secondary amine with sufficient aqueous stability to avoid competing with hydrolysis and tyrosine conjugation reactions. Applying an optimized resin containing this diazonium chemistry to HeLa cell lysates, they identified over 12,000 Kme1 sites across roughly 3700 proteins, an order-of-magnitude improvement over the prior

monomethyllysine proteome profiling methods. By utilizing isotope-labeling controls, the authors further revealed that a substantial fraction of the detected methylated proteins was not attributable to the canonical SAM-dependent pathway, pointing to the potential for alternative methylation mechanisms, opening the field to a novel direction of further study [143].

4.3. Applications of Cleavable Linker Strategies

The cleavable linker strategies discussed in Section 3.3 have also been extended beyond PTM site identification to small-molecule target engagement studies. The SEE-CITE (Silyl Ether Enables Chemoproteomic Interaction and Target Engagement) approach employs a cleavable silyl ether-functionalized drug analog to map covalent drug–protein interaction sites in native cellular contexts, mirroring the acid-cleavable design logic of the DADPS linker while substituting a small-molecule drug for a PTM-directed probe. This strategy successfully distinguished the binding sites of two kinase inhibitors, dasatinib and asciminib, that both target BCR-ABL1 but occupy distinct binding pockets, demonstrating the impressive precision achievable when cleavable enrichment chemistry is applied to chemoproteomics-based target engagement profiling [144].

Cleavable linker design has also been used to overcome a longstanding practical limitation of citrullination proteomics. The harsh elution conditions (boiling detergent or strong acids) required to release derivatized peptides from streptavidin resin have historically compromised peptide recovery and fragmentation quality, which is particularly damaging for citrulline studies as the expected mass shift is only around 1 Da. A recently reported workflow pairs an azido-phenylglyoxal handle, which reacts selectively with the citrulline ureido group, with a Dde-cleavable biotin-alkyne tag, allowing derivatized peptides to be released from streptavidin beads under mild hydrazine treatment rather than the harsh chemical or thermal elution that is usually necessary. Implemented in a 96-well high-throughput format, this workflow more than doubled citrullination site coverage in mouse brain relative to previously published probes. In addition, when applied to ionomycin-activated human neutrophils, it was revealed that across all core and linker histone proteins, site-specific citrullinated proteoforms were generated during neutrophil extracellular trap formation, with particularly pronounced regulation shown in linker histone H1 variants [107].

A complementary cleavable linker strategy addresses the reliance of most chemoproteomic workflows on conventional LC-MS/MS as the sole readout. A chemoselective butyraldehyde probe for protein lipoylation was coupled, via click chemistry, to a laser-cleavable gold nanoparticle reagent which allowed lipoylated proteins to be released and detected directly by laser desorption/ionization mass spectrometry. This in situ readout circumvents the standard requirement for on-bead tryptic digestion and downstream chromatographic separation, offering a faster alternative to the acid-, photo-, or reduction-cleavable chemistries discussed in Section 3.3 for applications prioritizing throughput over deep proteome coverage [145].

Finally, the DADPS cleavable-linker chemistry central to Section 3.3 has itself been re-engineered to enable quantitative, multiplexed chemoproteomics. The sCIP (silane-based Cleavable linkers for Isotopically labeled Proteomics) platform builds customizable, isotopically labeled, chemically cleavable biotin capture reagents while supporting click-assembled isobaric tags compatible with both MS1- and MS2-based quantitation. This addresses the high reagent cost and workflow complexity that have limited broader adoption of isobaric tandem mass tag strategies in cysteine- and PTM-directed chemoproteomics [146].

4.4. Applications of Proximity and Photoaffinity Labeling

The approach mentioned in Section 3.4 to characterize PTM-associated protein interactions, which employs photoactivatable crosslinking probes as opposed to enzymatic labeling, has been the proximity labeling technique with the most expansion. One approach exploits the site-selective photocrosslinking reactivity of sulfonium ions with tryptophan residues, allowing for the covalent capture of reader proteins. A synthetic histone H3 tail peptide was prepared in which the dimethylated lysine at K4 (H3K4me2) was replaced with a norleucine-ε-dimethylsulfonium analog that mimics the steric and electrostatic profile of the methyllysine mark. Upon UV irradiation, the sulfonium group formed a covalent crosslink with proximal tryptophan residues in interacting proteins with high selectivity, enabling the capture of H3K4me2 readers and co-associated protein complexes, including intact multi-subunit assemblies that would not survive conventional pulldown conditions [147].

A parallel strategy employs bifunctional lipid mimics bearing both a diazirine photocrosslinker and a terminal alkyne, where the diazirine enables UV-triggered covalent capture of proximal proteins participating in lipid-dependent interactions, while the alkyne serves as a handle for subsequent CuAAC-based enrichment or fluorescence visualization [148]. A third class of photoactivatable probes incorporates diazirine groups directly into synthetic arginine and lysine analogs for broad-spectrum histone PTM interactome profiling. These probes were applied to map the interactomes of H3R2me2a and H4K5acK8ac, and in a notable methodological integration, native chemical ligation (Section 3.5) was used to incorporate both the PTM and the diazirine crosslinker into reconstituted nucleosomes, enabling validation of identified interactions in a physiologically relevant chromatin context. This platform was further leveraged to characterize how H3R2me2a modulates the binding of the chromatin remodeling complex cBAF to said nucleosomes [149].

5. Outlook and Conclusions

The chemical biology toolkit described throughout this review reflects a field that has diversified considerably beyond its origins in bioorthogonal metabolic labeling. Metabolic mimics, chemoselective derivatization, cleavable enrichment linkers, proximity labeling, and genetic code expansion or chemical ligation each address a distinct point of failure in conventional PTM proteomics, whether that is the lack of a chromatographically distinctive handle, the unreliability of antibody recognition, the inability to localize a modified residue after enrichment, the transience of reader-protein interactions, or the need for a homogeneously modified protein for mechanistic study. Importantly, no single strategy is sufficient on its own; rather, the field's progress has come from recognizing how a given strategy fails at isolating a specific PTM and selecting, or engineering, the chemistry best suited to the specific task. This adjustable approach to isolating and studying PTM proteoforms is likely to remain the organizing principle of the field going forward, and future methodological progress will depend as much on the thoughtful combination of existing tools as on the invention of entirely new chemical biology tools.

This modular approach is a recurring theme across the applications discussed in Section 4, which highlights that the most powerful recent studies rarely rely on a single chemical biology strategy in isolation. The integration of chemoselective probes with genetic code expansion to simultaneously discover and functionally validate acetoacetylation sites [127], the combination of native chemical ligation with diazirine-based photocrosslinking to map PTM interactomes in a reconstituted nucleosomal context [133], and the pairing of pulse-chase metabolic labeling with secretome-specific sample preparation to resolve the temporal dynamics of itaconation [33] all illustrate a trend toward combinatorial platforms that exploit the complementary strengths of multiple tools within a single workflow. As more of these modular components become commercially available or broadly disseminated

through protocols, this combinatorial approach is being strengthened as the default strategy for characterizing newly discovered PTMs.

The continued growth of computational and AI-driven methods will further accelerate this trend. As highlighted in Section 1, deep learning-based de novo sequencing and open-search algorithms are already reducing the field's reliance on restrictive, modification-specific database searches, and this capability will become increasingly important as the number of chemically distinct PTMs exponentially outpaces the field's capacity to build dedicated search parameters for each one [38,39]. Beyond spectral interpretation, machine learning models trained on the growing collection of site-resolved PTM data hold considerable promise for predicting which residues, protein families, or sequence motifs are likely to harbor a given modification, allowing chemical biology efforts to be directed toward the most probable substrates rather than relying solely on unbiased discovery [40–42]. A less explored but promising future direction of the field is the application of generative and predictive modeling to probe design itself. For instance, this type of modeling could be utilized in anticipating whether a given bioorthogonal analog will be tolerated by a target enzyme's active site, which could reduce the substantial empirical optimization currently required to develop new metabolic labeling probes.

At the same time, several wet-lab methodologies discussed in this review remain comparatively underdeveloped relative to their apparent potential and warrant particular attention in future work. Intracellular chemical ligation via split-intein systems has thus far been demonstrated only as a proof-of-concept for histone tail modification [121], but the underlying strategy could in principle be extended to non-histone substrate proteins and to a broader range of ligation junctions, offering a route to site-specific PTM installation directly within a native cellular environment rather than in vitro. Similarly, enzyme engineering approaches that expand the substrate tolerance of biosynthetic machinery toward bulky bioorthogonal analogs have so far been applied to a limited number of enzyme families, such as the GalNAc-transferases discussed in Section 3.1, and systematic application of this strategy across other PTM-installing enzyme classes could substantially broaden the scope of metabolic labeling [28]. Photoaffinity and proximity labeling chemistries also stand to benefit from continued refinement of crosslinking radius and reader selectivity, building on recent advances such as Chroma-μMap [117], to further reduce background signal and improve confidence in identified PTM-protein interactions. Finally, cleavable linker chemistries compatible with acid-labile modifications such as O-glycans remain comparatively underdeveloped relative to the DADPS platform, and their advancement would extend site-level chemoproteomic profiling to PTM classes currently underserved by existing enrichment architectures.

Taken together, the chemical biology strategies reviewed here have already substantially expanded the set of PTMs amenable to systematic, proteome-wide characterization, and the continued convergence of these wet-lab tools with computational and AI-assisted analysis is poised to further accelerate discovery in the coming years. Realizing this potential will require sustained collaboration between synthetic and chemical biologists, mass spectrometrists, and computational scientists, but the trajectory of the field over the past decade suggests that many PTMs currently considered rare, low-stoichiometry, or poorly characterized are likely to be brought within reach in the near future [150].

Author Contributions: Conceptualization, Q.Z.; writing—original draft preparation, A.S.; writing—review and editing, X.M. and Q.Z.; visualization, A.S. and X.M.; supervision, Q.Z. All authors have read and agreed to the published version of the manuscript.

Funding: The research in Zheng Lab was financially supported by the NIH (R35 GM150676) and startup funds from Purdue University for Q.Z.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Acknowledgments: GPT-6 and Claude Sonnet 5.5 were utilized as an editor and citation search engine in this manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

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