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Recent advances in proximity labeling: New chemistries, optical control and programmable reaction boundaries

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Proximity labeling (PL) has become a broadly used chemical strategy for mapping molecular neighborhoods in living systems. Recent advances are shifting the field from expanding the PL toolbox toward engineering the reaction boundary that defines what is labeled, when labeling occurs, and how far reactive intermediates propagate. In this review, we highlight recent developments in four areas. First, new genetically encoded enzymatic systems reduce reliance on classical peroxide- or biotin-dependent chemistry. Second, optically controlled PL methods improve temporal gating through photoactivated enzymes, genetically encoded photocatalysts, self-labeling tag ligands and fluorogen-activating protein systems. Third, molecular ruler strategies and engineered enzyme-probe pairs are refining our understanding and control of labeling radius. Finally, emerging platforms repurpose covalent labeling for signal integration and functional amplification. Together, these advances position PL as a programmable chemical tool for spatial mapping, molecular recording and biological intervention.

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Introduction

Proximity labeling (PL) has matured from a specialized method for mapping local proteomes into a broad chemical strategy for recording molecular neighborhoods. Its central idea is simple: a genetically targeted catalyst generates a reactive intermediate *in situ*, and nearby biomolecules are covalently marked before the intermediate is quenched or diffuses away [1,2]. This design allows transient, weak, or spatially confined molecular associations to be captured in living cells without physical purification of the underlying structure.

As PL has been applied to increasingly diverse biological settings, the meaning of "proximity" has become more important and more complex. Spatial information is not determined only by where the PL catalyst is localized. It also depends on the chemistry, lifetime and diffusion of the reactive intermediate, the timing of activation, the accessibility of target residues or nucleobases, and the mobility of local biomolecules. Thus, two PL methods targeted to the same cellular site may report different molecular neighborhoods, not because one is necessarily more accurate than the other, but because they operate with different reaction boundaries.

Recent developments in PL have begun to address this issue directly. New enzymatic systems reduce reliance on classical peroxide- or biotin-dependent chemistry; light-controlled methods improve temporal gating and expand the range of reactive intermediates; molecular ruler strategies provide more quantitative views of labeling radius; and emerging platforms convert covalent labeling into a signal for recording, integration, or functional amplification. In this review, we focus on recent advances that illustrate this shift from expanding the PL toolbox to engineering the reaction boundary of proximity labeling.

Results

Expanding genetically encoded enzymatic PL chemistry

Because the reaction boundary of PL is set first by the chemistry that generates the reactive intermediate,

recent efforts have revisited the enzymatic core of PL. Classical enzyme-mediated methods have largely relied on two reaction strategies: peroxidase-catalyzed oxidation of phenol probes and biotin ligase-mediated activation of biotin. Recent developments have expanded this chemistry by introducing new enzymes, substrates and reactive intermediates that address limitations associated with peroxide toxicity, endogenous biotin background and poorly defined spatial reach.

One major direction is oxidative PL that reduces or avoids the need for exogenous hydrogen peroxide. Tyrosinase-based methods, such as TyroID, use molecular oxygen to oxidize phenol probes into quinone-like intermediates, enabling labeling under more biocompatible conditions [3,4] (Figure 1a). LaccID similarly relies on an engineered laccase to oxidize phenol substrates using oxygen as the terminal oxidant [5] (Figure 1b). In parallel, hydrogen peroxide-independent APEX2 strategies seek to preserve the utility of APEX-type oxidative labeling while minimizing perturbation from peroxide addition [6–10]. Although these methods differ in enzyme scaffold and reactive intermediate, they share the goal of maintaining efficient oxidative labeling while improving biological compatibility.

A second direction is to move away from endogenous biotin as the labeling substrate. Engineered lipoate ligase systems activate bioorthogonal small-molecule substrates rather than biotin, reducing background from endogenous biotin and expanding the chemical handles available for enrichment or detection [11,12]. In the recently reported FlexID system, engineered LplA variants promiscuously label neighboring proteins with synthetic substrates, providing a biotin-independent route to genetically encoded PL [13] (Figure 1c).

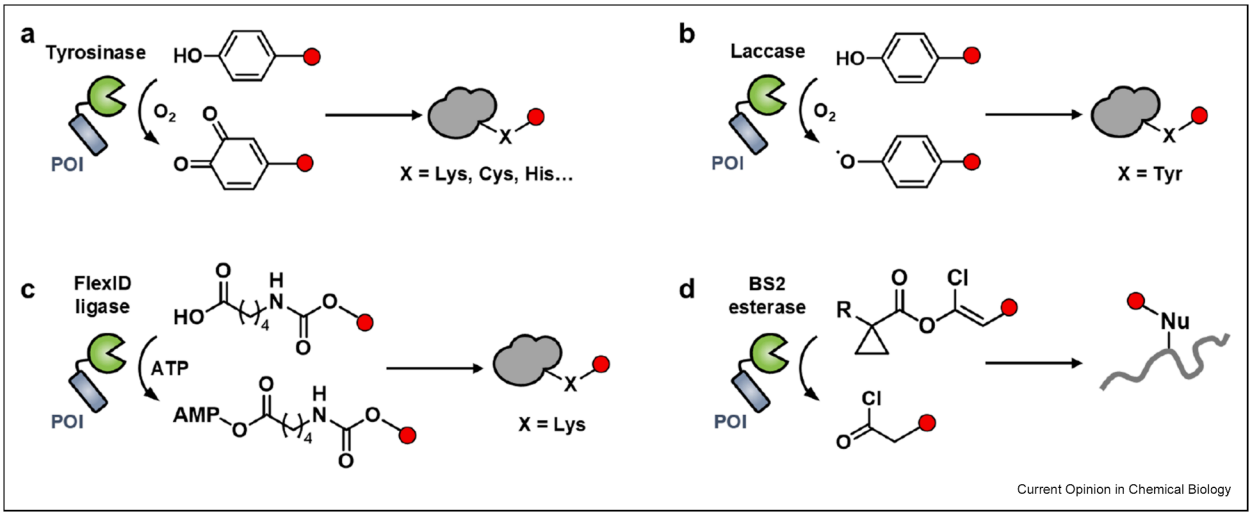
A third strategy uses enzymes to locally unmask reactive small molecules. BS2 esterase was first introduced in BAP-seq for RNA proximity labeling, where it converts a masked acyl chloride precursor into a reactive acylating species that modifies nearby RNAs [14] (Figure 1d). Although initially developed for transcriptome profiling, this acylation chemistry can also target proteins. Recent yeast-display evolution of BS2 identified improved variants using protein labeling as the selection readout, supporting its broader potential as a genetically encoded PL enzyme [15].

These advances move enzymatic PL beyond faster versions of APEX2 or TurboID. By changing the enzyme, substrate and intermediate, recent tools begin to tune compatibility, background and spatial reach. Yet chemical diversification alone does not define the labeling window, setting the stage for methods that control when labeling begins.

Optical control of PL reactions

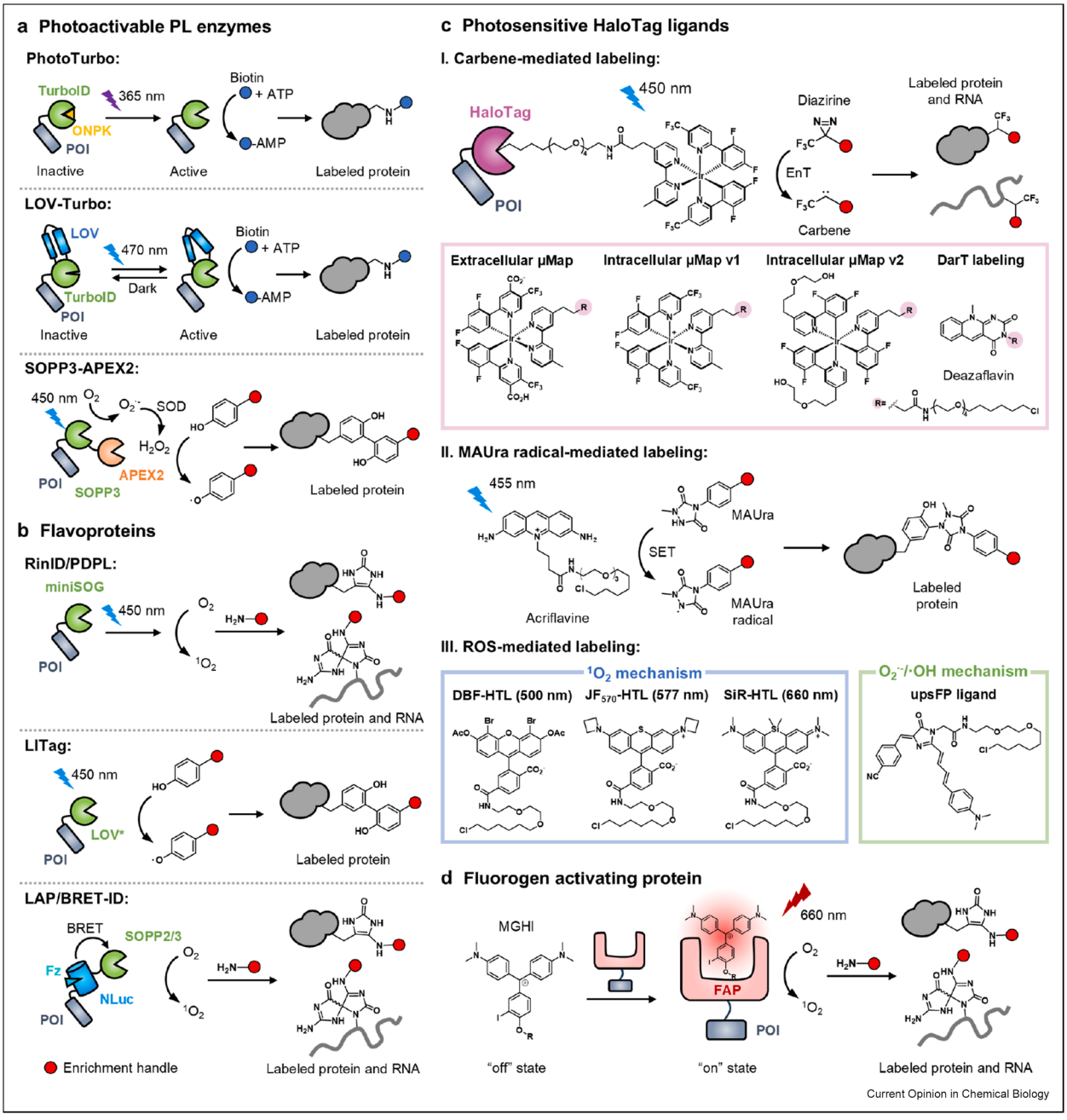
Changing the enzyme or substrate can tune what reactive intermediate is produced, but many biological processes also require control over when that intermediate is generated. Although APEX-mediated methods

Figure 1



Labeling mechanisms of emerging enzyme-based PL methods. (a) Tyrosinase oxidizes phenol substrates with molecular oxygen to generate quinone intermediates, which subsequently react with nucleophilic residues on neighboring proteins. **(b)** Laccase catalyzes the oxygen-dependent one-electron oxidation of phenol probes to generate phenoxyl radicals, which primarily label tyrosine residues. **(c)** Engineered lipoate protein ligase FlexID activates synthetic substrates bearing diverse chemical handles through an ATP-dependent mechanism, enabling covalent labeling of lysine residues. **(d)** BS2 esterase unmasks 1-methylcyclopropyl or phenylcyclopropyl esters to release reactive acylating species that can label nucleophilic biomolecules.

Figure 2



Genetically encoded photocatalytic proximity labeling platforms. (a) Photoactivatable PL enzymes. PhotoTurbo uses a photocaged catalytic lysine in TurboID (top). LOV-Turbo inserts a photosensitive LOV domain into TurboID (middle). SOPP3-APEX2 fuses SOPP3 to APEX2, allowing SOPP3-generated superoxide to form hydrogen peroxide and activate APEX2 labeling (bottom). **(b)** Flavoprotein-based PL. RinID and PDPL use amine probes to capture singlet oxygen-induced photooxidation products (top). LITag generates phenoxyl radicals for protein labeling (middle). LAP uses NanoLuc–SOPP2 and furimazine to drive bioluminescence-mediated singlet oxygen production in living animals (bottom). **(c)** HaloTag-directed small-molecule photocatalysts. Iridium complexes and flavin analogs convert diazirines into carbenes (I). Acriflavine generates MAUra radicals by single-electron transfer (II). Organic dyes generate reactive oxygen species (III). **(d)** Fluorogen-activating protein systems couple photosensitization to fluorogen binding, reducing background labeling.

enable rapid labeling, typically within 1 min, the onset of enzyme activation may lag behind hydrogen peroxide addition because it depends on small-molecule diffusion to the target site. Optical control addresses this temporal dimension of PL by allowing labeling chemistry to be activated with defined timing, spatial pattern and

wavelength. Recent strategies install optical control at several levels of the PL reaction.

One approach is to make established PL enzymes light responsive (Figure 2a). PhotoTurbo uses a photocaged catalytic lysine to suppress TurboID activity until light-induced uncaging restores biotin ligase function [16]. LOV-Turbo fuses TurboID to a LOV domain, whose blue-light-induced conformational change modulates access to the active site and enables light-regulated biotinylation [17]. SOPP3-APEX2 extends optical gating to peroxidase chemistry by using light-generated reactive oxygen species to initiate APEX2-mediated oxidative labeling, thereby replacing direct peroxide addition with light-dependent oxidative labeling [7]. These methods preserve familiar PL reactions while improving temporal control.

A second class uses genetically encoded photocatalysts as the labeling module (Figure 2b). RinID/PDPL and LITag generate reactive oxygen species or radicals under illumination, enabling local labeling of proteins or RNAs with precise temporal control [18–21]. Lantern, an engineered flavoprotein optimized by directed evolution, further improves this strategy by increasing labeling efficiency and enabling rapid mapping of subcellular transcriptomes and proteomes [22]. Both LITag and Lantern-mediated CAP-seq achieve temporal resolution down to 1 s, which is difficult to attain with traditional enzyme-mediated PL methods. LAP and BRET-ID replace external illumination with bioluminescence-driven activation, which may improve compatibility with *in vivo* settings where light delivery is challenging, although with less precise temporal control [23,24]. In these systems, genetic targeting defines the source of photochemistry, whereas the activation mode determines the labeling window.

Optical PL can also be built from small-molecule photocatalysts. Because antibody-, peptide- and small-molecule-targeted photocatalytic PL approaches have been excellently reviewed elsewhere [25,26], we focus here on strategies that recruit photocatalysts through genetically encoded protein tags. HaloTag-based systems use covalently bound ligands bearing transition-metal photocatalysts, as exemplified by μMap chemistry [27,28], or synthetic chromophores such as acriflavine [29], dibromofluorescein [30,31], JF_{570} (POCA) [32], silicon rhodamine (SeeID) [33] or upsFP ligand [34], thereby placing photochemistry at user-defined sites (Figure 2c). These designs are modular: changing the ligand can alter activation wavelength, reactive intermediate and biomolecule preference without reengineering the protein tag. However, untargeted or incompletely washed ligand may contribute background, motivating fluorogenic or target-activated designs such as SeeID [33].

Fluorogen-activating protein systems provide another route to target-activated photocatalysis by coupling photosensitization to ligand binding (Figure 2d). In FAP-seq, FAP-bound monoiodinated malachite green acts as a near-infrared photosensitizer for RNA proximity labeling [35]. FLAPP uses a related FAP-malachite green system for genetically encoded near-infrared protein labeling [36]. These methods combine chemical tunability, fluorogenic activation and longer-wavelength illumination, suggesting a route toward lower-background optical PL.

Light-controlled PL extends reaction-boundary engineering into the time domain by defining the onset, duration and spatial pattern of catalyst activation. However, controlling when a reactive intermediate is generated does not fully define how far it travels, making labeling radius the next critical parameter.

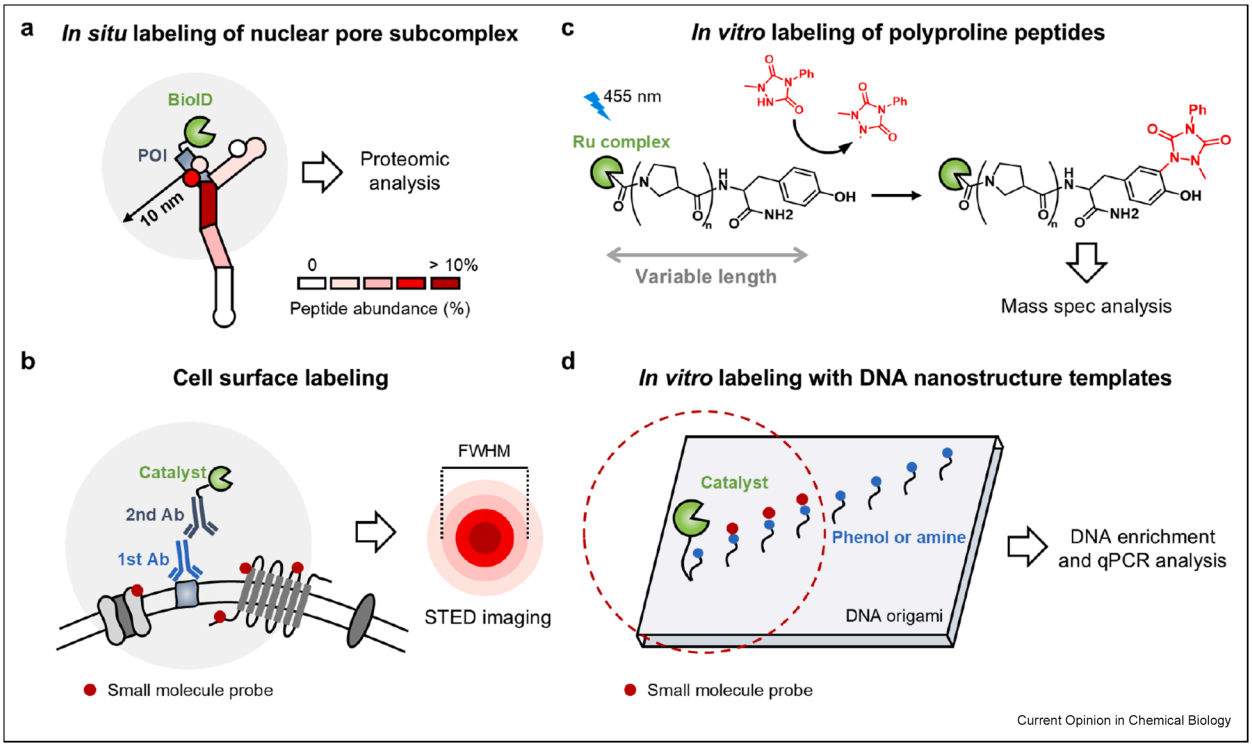
How proximal is proximity labeling?

Even when the timing and location of catalyst activation are precisely controlled, the spatial meaning of PL remains governed by how far the resulting reactive intermediate or enzyme-bound substrate can propagate. This has brought renewed attention to a deceptively simple question: how proximal is proximity labeling? Early descriptions often assigned each PL enzyme a nominal labeling radius, but recent studies indicate that this value should not be treated as an intrinsic constant. The effective reaction boundary depends on the lifetime and diffusion of the reactive intermediate, enzyme–substrate interactions, local quenching, residue accessibility, labeling duration, and mobility of both the enzyme and target biomolecules. Thus, PL produces a spatially weighted chemical map rather than a sharp molecular boundary.

Several strategies have been developed to estimate the reaction boundary of PL. In cells, nuclear pore subcomplexes provide a useful calibration system because their architecture is well defined and their protein components occupy distinct positions within a stable assembly [37] (Figure 3a). Cell-surface labeling assays offer another cellular approach by placing enzyme and acceptor molecules at controlled membrane distances or across defined cell–cell interfaces [38] (Figure 3b). These cell-based assays preserve relevant biological environments, but their interpretation can be influenced by protein mobility, local accessibility and membrane geometry.

More reductionist *in vitro* systems provide complementary information by isolating reaction geometry from the complexity of living cells. Intramolecular labeling through polyproline linkers of different lengths has been used to estimate the spatial reach of small-molecule photocatalyst-based PL [39] (Figure 3c). DNA-origami molecular rulers further improve geometric control by

Figure 3



Measuring the reaction boundary of PL platforms. (a) BioID reaction boundary estimated by *in situ* labeling of nuclear pore subcomplexes followed by spatial proteomics. **(b)** Cell-surface labeling assays combine defined membrane geometry with super-resolution imaging to infer PL reaction boundaries in cells. **(c)** MAUra radical reaction boundary measured using synthetic polyproline peptides of defined lengths. **(d)** DNA nanostructure-based molecular rulers position PL enzymes and substrates at programmed distances to quantify reaction boundaries *in vitro*.

positioning PL enzymes and protein substrates at defined nanometer-scale distances, while encoding distance information into sequencing readouts for multiplexed radius measurement [40] (Figure 3d). This strategy enables precise control of the enzyme–substrate distance and quantification of labeling efficiency at defined distance intervals. However, *in vitro* systems do not fully recapitulate the cellular context. Molecular crowding and endogenous quenchers in cells may limit the diffusion of reactive intermediates, resulting in a smaller labeling radius *in vivo* than that measured *in vitro*. In addition, phenol groups anchored on DNA origami may not fully represent solvent-exposed tyrosine residues on protein surfaces. Collectively, these approaches show that labeling radius is experimentally measurable, but strongly dependent on both reaction chemistry and assay context.

These measurements have also clarified mechanistic differences between PL enzymes. TurboID has often been assumed to label through diffusion of released biotin-AMP. However, recent radius measurements indicate that TurboID labeling occurs mainly through a contact-dependent mechanism, in which productive labeling requires close enzyme–substrate encounter [40]. By contrast, APEX2 appears to combine contact-dependent labeling with diffusive labeling mediated by phenoxyl radicals [40]. This distinction helps explain why different PL methods can generate related but nonidentical molecular maps even when targeted to the same cellular site.

An important trend is therefore not only to measure labeling radius, but also to engineer it. The FlexID system represents a contact-labeling strategy, using engineered LplA chemistry to achieve zero-length or near-contact-dependent labeling [13]. Radius engineering has also been pursued by tuning peroxidases and their cognate probes. ^{L242F}APEX2 was identified by directed evolution toward biotin-aniline, enabling faster labeling while maintaining spatial specificity [41]. Separately, Martell and coworkers developed APOX to oxidize nitrophenol-based substrates [42]. These enzyme-probe combinations generate more reactive, short-lived radicals that are rapidly quenched during diffusion, thereby reducing the effective labeling footprint. Such radius-restricted methods can improve interpretability for direct molecular contacts, although

they may reduce coverage for broader subcellular environments.

Importantly, the optimal reaction boundary is not always the smallest one. MultiMap takes advantage of PL reactions operating at multiple length scales to infer protein–protein interactions from differential labeling patterns [43]. This approach reframes labeling radius as a tunable source of spatial information rather than merely a limitation. More generally, as PL reactions become more controllable in chemistry, timing and spatial reach, the covalent mark deposited by PL can be used not only to identify neighboring molecules, but also to record or amplify biological events.

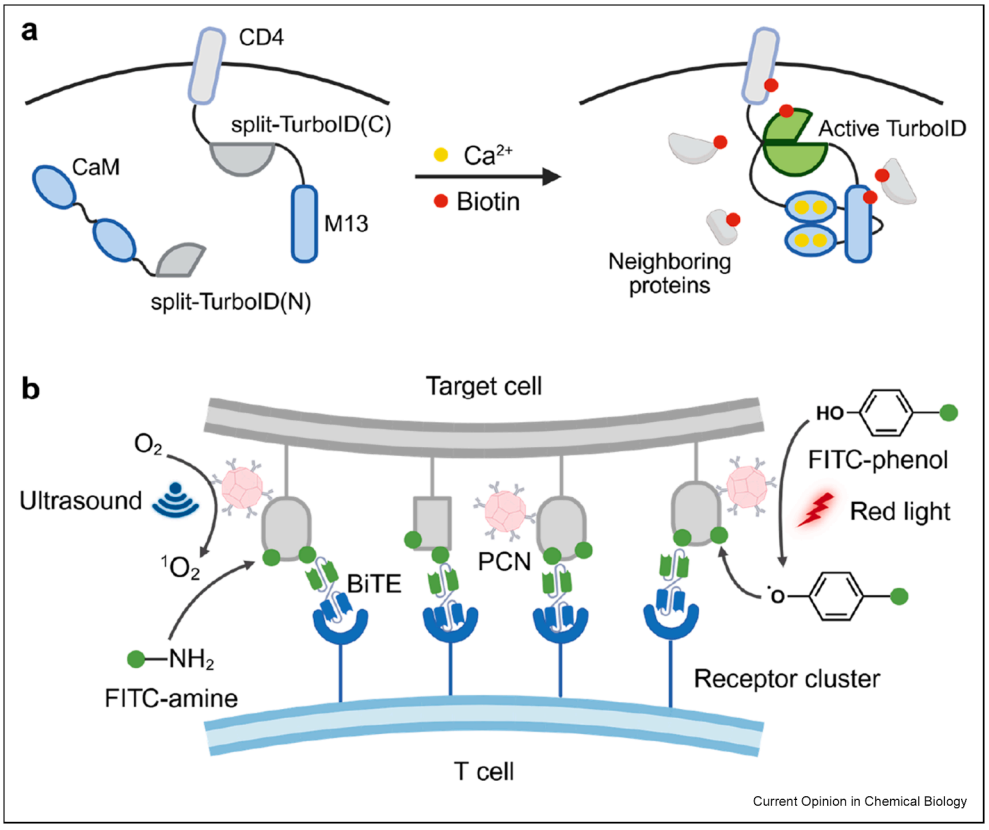
PL beyond mapping

PL is increasingly moving beyond identifying nearby biomolecules toward converting proximity into a durable biological signal. Because the covalent mark persists after the original molecular encounter has ended, PL can record transient or cumulative events that would otherwise be difficult to capture by imaging or biochemical isolation.

One emerging direction is PL-based signal integration. Cal-ID and CaST both convert calcium activity into cumulative biotin labeling, but through distinct mechanisms: Cal-ID uses calcium-dependent conformational changes to drive refolding of circularly permuted TurboID [44], whereas CaST relies on calcium-dependent reconstitution of split TurboID [45] (Figure 4a). Similar principles have been applied to receptor signaling, where GPCR-arrestin association is coupled to biotinylation through split TurboID reconstitution [46] or BRET-induced LOV-Turbo activation [17]. In principle, such architectures could be paired with different triggers, substrates or downstream readers to record signaling history, cell–cell contact, organelle proximity or protein assembly.

PL can also be repurposed for functional amplification. PATCH uses proximity labeling to amplify antigen recognition for cancer immunotherapy, converting a local target-binding event into enhanced immune activation [47] (Figure 4b). This strategy illustrates a broader opportunity: the product of PL does not have to serve only as an analyte for mass spectrometry or

Figure 4



Emerging applications of PL beyond molecular mapping. (a) Schematic of CaST for calcium-signal integration. Elevated intracellular calcium drives split-TurboID reconstitution, leading to biotinylation of proximal proteins in the presence of biotin. **(b)** PL-based amplification of antigen-induced cellular responses. Under red-light or ultrasound stimulation, target cell-anchored porous coordination networks (PCNs) covalently tag fluorescein probes on the cell surface. The resulting high-density fluorescein signals cluster and direct a fluorescein-binding bispecific T cell engager (BiTE), thereby enhancing T cell activation and cytotoxicity. The schematics were created using BioRender.

Table 1

Overview of proximity labeling techniques.

PL Methods	Reactive intermediate	Labeling duration	Substrate specificity	Labeling radius
Conventional enzyme-mediated PL				
Peroxidase-mediated PL (APEX, APEX2, HRP)	Phenoxyl radical	1 min	Protein: Tyrosine and other electro-rich residues RNA: Guanosine	269 nm (*in vitro*) [38,40]
Biotin ligase-mediated PL (BioID, BASU, TurboID)	Biotinyl-5′-AMP	10 min-18 h	Protein: Lysine	Contact-dependent [40]
Recently developed enzyme-mediated PL				
Tyrosinase-based PL (TyroID)	*o*-quinone	1–30 min	Protein: Cysteine, lysine, histidine and other nucleophilic residues	Longer than phenoxyl radical [4]
Laccase-based PL (LaccID)	Phenoxyl radical	1–2 h	Protein: Tyrosine and other electro-rich residues	269 nm (*in vitro*) [38]
Lipoic acid ligase-based PL (FlexID)	Lipoyl-AMP	5 min	Protein: Lysine	Contact-dependent [13]
BS2 esterase-mediated PL (BAP-seq)	Acylating agents (e.g. acid chloride)	3–10 min	Nucleophilic sites on RNAs and proteins	–
Photocatalytic PL				
$^1O_2$-mediated PPL (e.g., CAP-seq, Halo-seq, FAP-seq, RinID, PDPL, etc.)	$^1O_2$	15–20 min	Protein: Histidine RNA: Guanosine	70 nm (*in vitro*) [48]
μMap	Carbene	5 min	Protein: All residues RNA: All nucleosides	54 nm (*in vitro*) [38]
LITag	Phenoxyl radical	1–3 s	Protein: Tyrosine	269 nm (*in vitro*) [38]
upsFP	•OH and $O_2^{\bullet-}$	20 min	Protein: Histidine, glutamic acid, phenylalanine, and tryptophan	<100 nm (*in vitro*) [34]
iPPL	MAUra radical	1 min	Protein: Tyrosine	<10 nm (*in vivo*) [29,39]

sequencing. It can become an input for recruitment, activation, enrichment or therapeutic response.

These examples point to a transition from PL as a descriptive omics method to PL as a programmable chemical operation. Once the reaction boundary is defined and the covalent mark can be coupled to an appropriate reader, proximity labeling can record, integrate or amplify biological events with molecular specificity. This functional turn will likely become an important direction for the next generation of PL technologies.

Outlook

Taken together, these advances suggest that PL development will be defined less by the number of proteins or RNAs detected and more by how precisely the labeling reaction can be controlled. Important parameters include the identity and lifetime of the reactive intermediate, activation kinetics, biomolecule scope, labeling radius, background reactivity, and compatibility with downstream readouts (Table 1). These features should ideally be measured under conditions that match the intended biological application, because the same PL chemistry may behave differently in purified systems, cultured cells, tissues or living animals.

The most useful PL method will not always be the one with the strongest signal or smallest radius. Instead, it will be the one whose reaction boundary best matches the biological question. Mapping a subcellular environment, identifying a direct molecular contact, recording a transient signaling event and amplifying a therapeutic recognition signal all require different balances among sensitivity, spatial reach, timing and perturbation. As PL becomes increasingly engineerable, its future impact will depend on choosing and designing chemistries with the right boundary for the right biological problem.

Beyond selecting suitable PL methods, current studies highlight the importance of controlling background noise through ratiometric comparisons with appropriate reference controls, such as input, unlabeled, and untargeted controls. Given the complexity of biological systems, orthogonal validation is also recommended to minimize false positives.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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