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Proximity-dependent labeling methods for proteomic profiling in living cells: An update

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Abstract

Characterizing the proteome composition of organelles and subcellular regions of living cells can facilitate the understanding of cellular organization as well as protein interactome networks. Proximity labeling-based methods coupled with mass spectrometry (MS) offer a high-throughput approach for systematic analysis of spatially restricted proteomes. Proximity labeling utilizes enzymes that generate reactive radicals to covalently tag neighboring proteins. The tagged endogenous proteins can then be isolated for further analysis by MS. To analyze protein–protein interactions or identify components that localize to discrete subcellular compartments, spatial expression is achieved by fusing the enzyme to specific proteins or signal peptides that target to particular subcellular regions. Although these technologies have only been introduced recently, they have already provided deep insights into a wide range of biological processes. Here, we provide an updated description and comparison of proximity labeling methods, as well as their applications and improvements. As each method has its own unique features, the goal of this review is to describe how different proximity labeling methods can be used to answer different biological questions.

This article is categorized under:

Technologies > Analysis of Proteins

KEYWORDS

APEX, BioID, HRP, proximity labeling PUP-IT

1 | INTRODUCTION

Specialized biological processes occur in different organelles and subcellular regions. In addition, protein functions correlate with their subcellular localizations and interactions. Understanding how cellular structures underlie specialized functions requires the comprehensive identification of proteins within spatially defined cellular domains. Further, identification of interacting proteins is key to elucidating the mechanisms underlying complex cellular processes.

Mass spectrometry (MS) techniques have been used to systematically characterize the proteome of isolated organelles and protein interactors purified by affinity pull-down or following crosslinking. However, these approaches are limited by available purification methods, as it is not possible in many cases to obtain intact organelles of high purity. Moreover, even when purification is possible, contamination that results in false positive identification is common. For example, false positives may be introduced by cellular disruption, as two proteins that normally localize in different

subcellular regions may artificially interact when membranes are disrupted. In addition, false negatives often occur due to loss of components caused by disruption of isolated organelles or protein complexes. Additionally, a variety of discrete cellular regions cannot be purified by centrifugation, such as specialized endoplasmic reticulum (ER)-plasma membrane (PM) junctions that are critical for lipid metabolism and Ca^{2+} signaling (Carrasco & Meyer, 2011; Elbaz & Schuldiner, 2011; Hogan, Lewis, & Rao, 2010; Stefan, Manford, & Emr, 2013). Similarly, transient or weak interactions may be lost during purification of a protein interactome due to stringent washes.

Recently, proximity-dependent labeling methods have been developed and utilized for mapping compartmental proteome and protein interactomes. In this updated review, we compare proximity labeling techniques that utilize different enzymes and describe how they are used to address limitations of traditional methods.

2 | OVERVIEW OF ENZYME-CATALYZED PROXIMITY LABELING FOR PROTEOMIC PROFILING

In general, proximity labeling relies on enzymes that convert a substrate into a reactive radical that covalently tags neighboring proteins. We will discuss four major enzyme systems utilized for proximity labeling: BioID (proximity-dependent biotin identification), HRP (horseradish peroxidase), APEX (engineered ascorbate peroxidase), and PUP-IT (pupylation-based interaction tagging).

To achieve spatially restricted labeling, the enzymes are usually fused with a targeting signal peptide, a protein of interest, or antibody. After performing proximity labeling in living cells, cells are then lysed and tagged endogenous proteins are isolated using streptavidin beads. Small peptides from enriched proteins are generated by trypsin digestion and subsequently analyzed by tandem mass spectrometry (aka MS/MS or MS^2). The mass-to-charge (m/z) ratio of peptides and their fragment ions are then used to identify the peptide sequence through computational comparison against an established database (Figure 1).

Importantly, with proximity labeling, cells and tissues remain intact when the proteome or interactome is labeled. Thus, the potential for false-positive identifications is minimized, as artificial interactions caused by disruption of cells and contaminants during purification steps no longer affect the results. Moreover, proximity labeling can be applied to bypass organelle purification steps, offering an alternative approach for systematic proteomic characterization in live cells. As proximity labeling is an emerging method that enables proteomic profiling of organelles, subcellular domains, and interactomes, this updated review aims to provide an overview of the different methods to aid planning and execution of future experiments.

3 | BIOID-BASED PROXIMITY LABELING

BioID-based proximity labeling employs a mutant form of the biotin ligase BirA from *Escherichia coli* (Choi-Rhee, Schulman, & Cronan, 2004; Cronan, 2005; Roux, Kim, Raida, & Burke, 2012). The biotin ligase BirA is a conserved enzyme that mediates the attachment of biotin to target proteins (Chakravartty & Cronan, 2012). In the presence of ATP, BirA biotinylates proteins by catalyzing the conversion of biotin to reactive biotinoyl-5′-AMP, which specifically tags a lysine residue of a subunit of the acetyl-CoA carboxylase (Chapman-Smith & Cronan Jr., 1999; Choi-Rhee et al., 2004). Wild-type BirA has a high affinity to biotinol-5′AMP and keeps it in the active site until the acetyl-CoA carboxylase, or a short acceptor peptide, becomes available (Beckett, Kovaleva, & Schatz, 1999). Since BirA has a high specificity for its target sequence, it has been used to study specific protein–protein interactions (Fernandez-Suarez, Chen, & Ting, 2008): BirA is fused to a bait protein and BAP (biotin acceptor peptide) is fused to a prey protein. If the interaction occurs, the prey will be close enough to the bait to become biotinylated.

To achieve promiscuous labeling, the active site of BirA has been mutated, enabling random biotinylation of vicinity proteins without BAP (Choi-Rhee et al., 2004; Cronan, 2005). This method is named BioID and the mutated form of BirA for proximity labeling is called BioID or BirA* to be distinguished from the wild-type and other mutant forms of BirA (Roux et al., 2012) (Figure 2 and Figure 3). When the active site of BirA is mutated (R118G), its affinity to biotin-5′AMP is greatly reduced. The highly-reactive biotinoyl-5′-AMP is released from the active site of BioID and nonspecifically reacts with nearby proteins. Therefore, BioID can covalently tag nearby endogenous proteins on lysine residues. Although the labeling radius of BioID may vary depending on the local environment, the labeling radius of

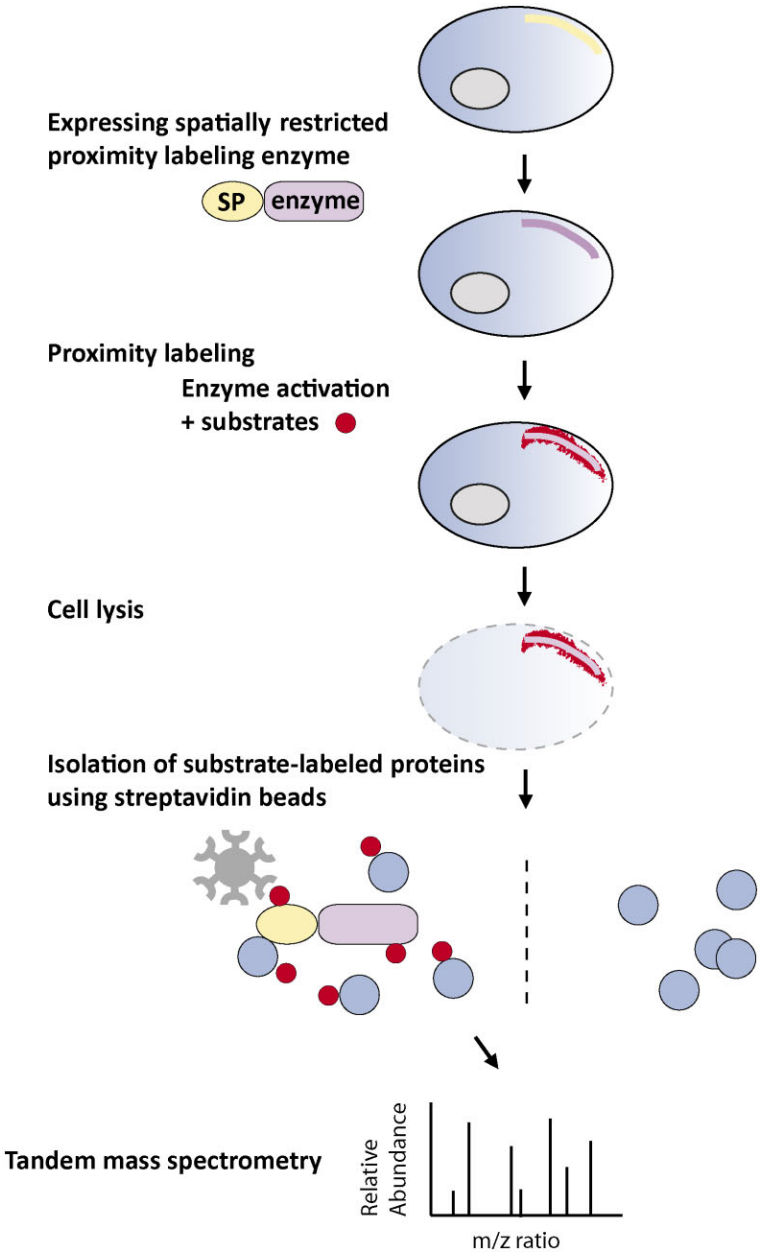
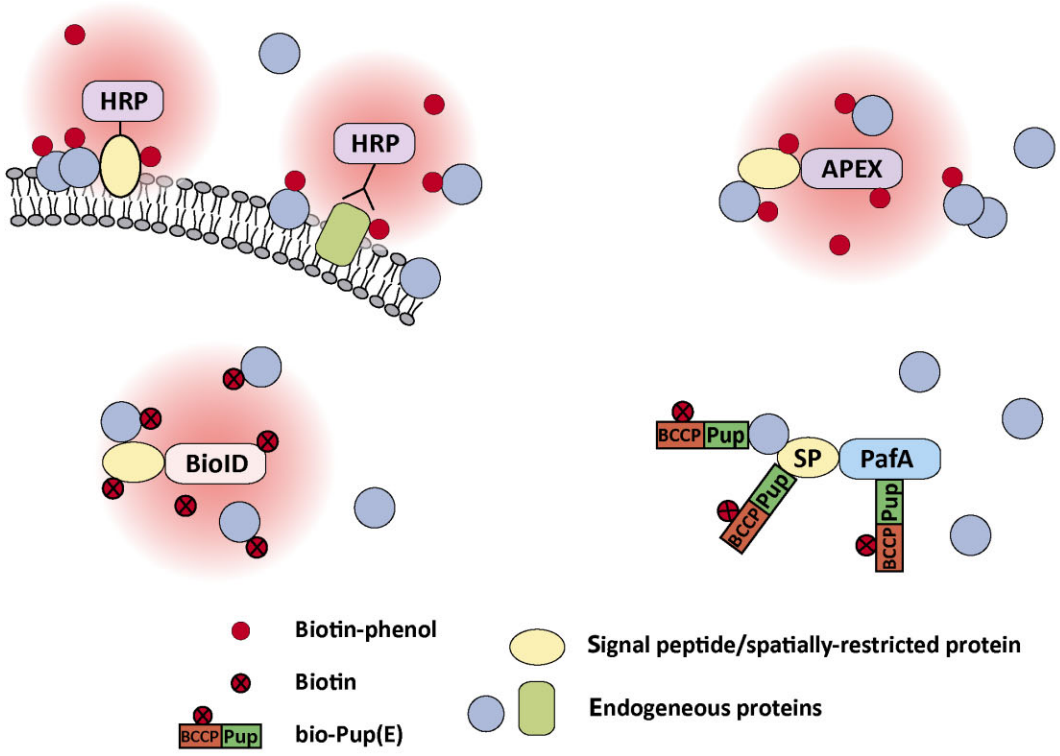


FIGURE 1 Proximity labeling for proteomic profiling. To achieve regional protein labeling, the enzymes are usually fused with a targeting signal peptide or a spatially restricted protein (SP). The enzymes can also be fused with any protein of interest for protein interactome studies. After performing proximity labeling in living cells, the cells are lysed and the tagged endogenous proteins are isolated using steptavidin beads. Small peptides of enriched proteins are generated by trypsin digestion and subsequently ionized for tandem mass spectrometry (MS/MS) analysis. The mass-to-charge (m/z) ratio of each peptide and their fragment ions is then used to identify peptide sequence through computational comparison against established databases

BioID is estimated to be around 10 nm using the structure of the nuclear pore complex as a "molecular ruler" (Kim et al., 2014).

In addition to the *E. coli* BioID enzyme, promiscuous biotin ligases from other species have been isolated. BioID2 was generated with an R40G mutation in the reactive site of a biotin ligase from *Aquifex aeolicus* to allow promiscuous labeling (Kim et al., 2016). BioID2 lacks the DNA binding domain at the N-terminus and is thus smaller (233 a.a.) than *E. coli* BioID (321a.a.), potentially minimizing functional interference with a tagged protein. BioID2 performs similar labeling chemistry as BioID but shows a higher activity and requires less biotin. Similarly, BASU is a promiscuous BirA from *Bacillus subtilis* with improved biotinylation activity compared to BioID and BioID2 (Ramanathan et al., 2018). Like BioID2, BASU lacks the N-terminal DNA-binding domain and is smaller than BioID. Finally, ancestral reconstruction of BirA proteins led to the recent isolation of a promiscuous biotin ligase called AirID, which exhibits robust biotinlyation in cultured human cells (Kido et al., 2020).

The BioID enzyme has also been engineered for increased activity. TurboID was isolated by directed evolution of BioID for increased biotinlyation activity via yeast display (Branon et al., 2018). In human HEK293T cells, TurboID can label an equivalent amount and diversity of proteins in 10 minutes as BioID, BioID2, or BASU can label in 18 hr. A smaller variant of TurboID called miniTurbo lacks the DNA-binding domain while still retaining robust biotinylation



	HRP	APEX	BioID	Pup-IT
Enzymatic Activity	Peroxidase	Peroxidase	Biotin ligase	Pup ligase
Labeling Target	Electron-rich amino acids	Tryrosine and potentially other electron-rich amino acids	Lysine	Lysine
Size	44 kD	27 kD	BioID, TurboID: 35kD BASU: 29kD miniTurbo: 28kD BioID2: 27kD	54kDa
Labeling time	5-10min	1min	BioID:15-24hr TurboID: 10min	24hr
Incubation time with substrate	5-10min	30-60min	BioID:15-24hr TurboID: 10min	24hr
Activation by H_2O_2	Yes	Yes	No	No
Substrates for protein labeling	biotin-phenol (and biotin- or fluorescein-acylazide)	biotin-phenol	biotin	bio-PupE bio-DE28 bio-Peptide4.1
Half-life of generated radicals	<1ms	<1ms	mins	Phosphorylated Pup(E) intermediate remains bound to PafA
Active region	extracellular, secretory pathway (inactive in cytosol)	intracellular	intracellular	intracellular, extracellular
Notes	Can be used as EM tag; HRP-conjugated antibodies available	Can be used as EM tag	BioID: reduced activity below 37°C TurboID: evolved in yeast at 30°C	

FIGURE 2 Proximity labeling methods. HRP and APEX are peroxidases that, when activated by H_2O_2, are able to turn biotin-phenol substrates into highly reactive radicals that covalently tag neighboring proteins on electron-rich amino acids. HRP is inactive in a reducing environment, such as the cytosol, but functions extracellularly. BioID, a mutant form of the biotin ligase BirA, can convert biotin into radicals that can covalently tag neighboring proteins on lysine residues. PafA is a ligase that can covalently tag neighboring proteins with the small protein Pup onto lysine residues. APEX, engineered ascorbate peroxidase; BioID, proximity-dependent biotin identification; HRP, horseradish peroxidase

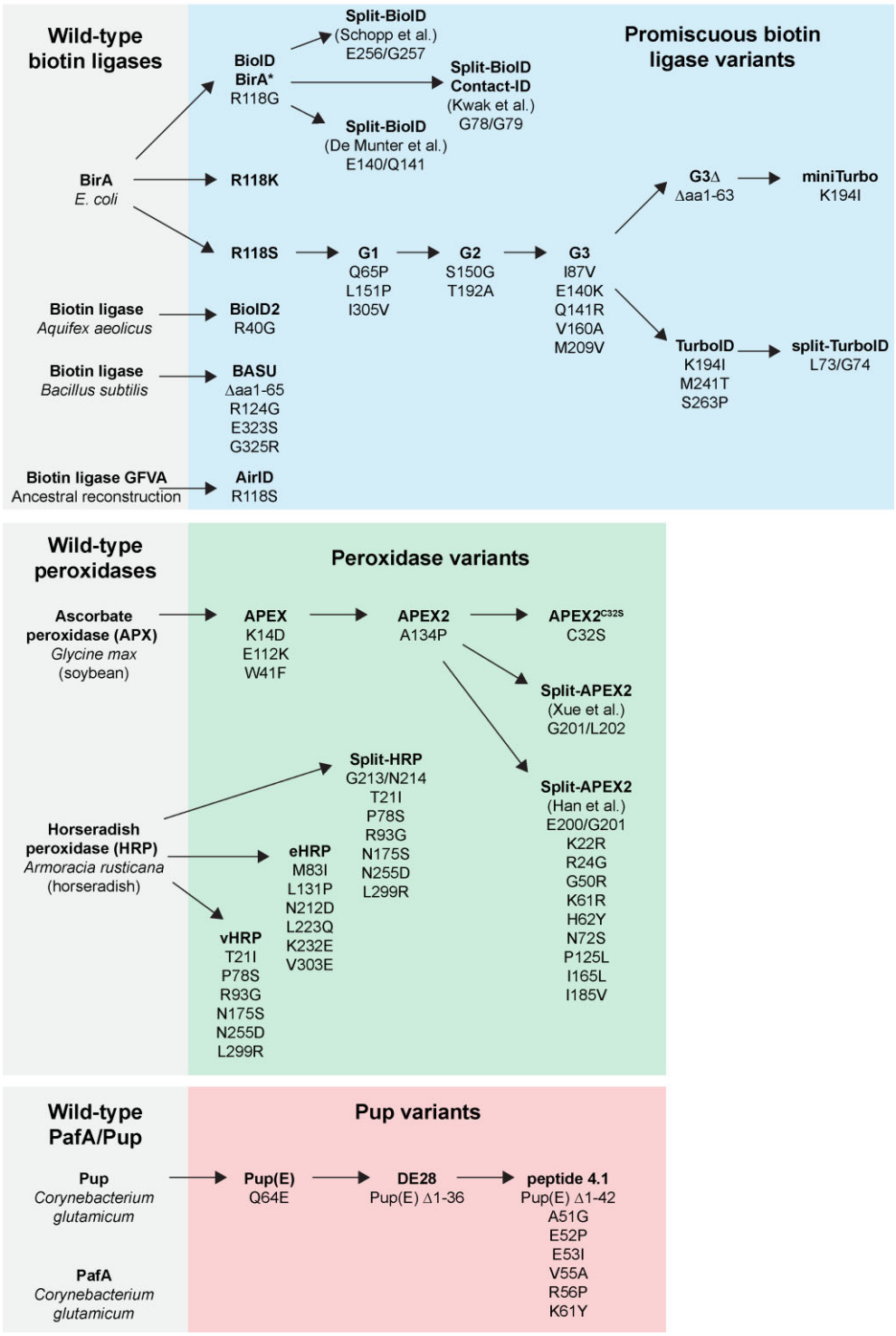


FIGURE 3 Directed evolution of proximity labeling components. Proximity labeling enzymes have been modified from their wild-type counterparts by selecting for mutants with promiscuous activity. Directed evolution has been used to isolate enzymes with increased activity, increased stability, smaller molecular weight, and that are split into inactive fragments that reconstitute activity when combined. Smaller Pup substrates have also been identified

activity. While miniTurbo has ~2x fold less activity than TurboID, it exhibits lower biotinlyation activity in the absence of exogenously added biotin and thus may be more suited to tighter labeling windows. Under extreme conditions (e.g., high expression levels, long labeling times), TurboID expression can be toxic in human cells, flies, and worms, suggesting that the evolution of this enzyme for increased activity may have effectively reached an upper limit. In addition to being useful in spatial proteomics (Branon et al., 2018), TurboID has also proven successful to discover new protein–protein interactions (Larochelle, Bergeron, Arcand, & Bachand, 2019; Mair, Xu, Branon, Ting, & Bergmann, 2019; Tachie-Menson et al., 2020; Zhang et al., 2019). However, for some bait proteins, TurboID may

increase the number of labeled background proteins relative to BioID (May, Scott, Campos, & Roux, 2020), perhaps due to its robust enzymatic activity.

Promiscuous biotin ligases have also been engineered with new functions. By screening all possible mutations at R118 in *E. coli* BirA, a new promiscuous biotin ligase variant (R118K) was isolated (Oostdyk et al., 2019). While R118K activity was less than R118G (BioID), R118K may be useful for proximity labeling under conditions where exogenous biotin is not added. Three independent studies derived split-BioID proteins, which were identified by screening for inactive fragments of BioID that can reform to restore biotinylation activity when physically brought together (De Munter et al., 2017; Kwak et al., 2020; Schopp et al., 2017). By linking these BioID fragments to two interacting proteins, the split-BioID system can be used to label proximal proteins only associated with this protein–protein pair. Recently, a split-TurboID system has been developed, with more robust labeling upon reconstitution (Cho et al., 2020).

Promiscuous biotin ligase enzymes has been used to map local interactomes, identify transient protein interactions, map organelle components, and thus provide a better understanding of cellular structures as well as interactions occurring during signal transduction. The application and impact of promiscuous biotin ligases have been extensively reviewed (Kim & Roux, 2016; Li, Li, Wang, & Di, 2017; Varnaite & MacNeill, 2016). Recent applications include interaction mapping of Ras (Kovalski, Shanderson, & Khavari, 2019), mitochondrial transcription elongation factor (Jiang et al., 2019), influenza A virus PA-X (Gaucherand et al., 2019), growth factor independence 1B (McClellan et al., 2019), receptor PTPRK (Fearnley et al., 2019), murine coronavirus replicase transcriptase complex (V'Kovski et al., 2019), PCNA (Srivastava et al., 2018), NHLRC2 (Paakkola et al., 2018), GRPEL1/2 (Konovalova et al., 2018), and IGF1R (Bareja, Hodgkinson, Soderblom, Waitt, & Dzau, 2018), *Toxoplasma gondii* conoid proteins (Long, Anthony, Drewry, & Sibley, 2017), N-cadherin (Li et al., 2019), the NuRD complex (BioID2) (Sher et al., 2019), plant N immune receptor (Zhang et al., 2019), protein arginine methyltransferase Rmt3 and the RNA exosome subunits, Rrp6 and Dis3 (Larochelle et al., 2019), AKAP18 (Smith et al., 2018), plant transcription factor FAMA (Mair et al., 2019), and stress granules (SGs) processing bodies (PBs) (Youn et al., 2018), and desmosomes (Badu-Nkansah & Lechler, 2020). In addition, BioID has recently been used to identify RNA-binding proteins by tethering BioID to RNA transcripts via MS2 aptamers (Mukherjee et al., 2019), and used in conjunction with traditional affinity purification to improve proteomic coverage and help determine distances between protein complex members (Liu et al., 2018).

4 | HRP-BASED PROXIMITY LABELING

HRP is a peroxidase that, when activated by H_2O_2, is able to convert a substrate into a highly reactive radical that covalently tags neighboring proteins on electron-rich amino acids (Li et al., 2014). HRP is inactive in a reducing environment, such as the cytosol, because the structure of HRP, which is maintained with four disulfide bonds and two Ca^{2+} ion-binding sites, is disrupted in reducing conditions (Hopkins, Gibson, Stinchcombe, & Futter, 2000). This has limited its use for determining intracellular interactomes, and motivated the development of APEX. Nevertheless, HRP is active in oxidizing environments, such as the lumen of the ER or the Golgi and the extracellular region. Thus, HRP has been used for proteomic mapping on the surface of living cells (Cijsouw et al., 2018; Li et al., 2020; Loh et al., 2016; Wu, Nagala, & Crocker, 2017). In addition, HRP can also be used as an electron microscopy (EM) tag (Ellisman, Deerinck, Shu, & Sosinsky, 2012). With H_2O_2, HRP can catalyze the polymerization of 3,3′-diaminobenzidine (DAB) which precipitates and creates an EM contrast after OsO_4 fixation.

Although HRP can catalyze a variety of substrates, for proximity labeling two in particular have been used: (a) the enzyme-mediated activation of radical source (EMARS) method uses fluorescein arylazide or biotin arylazide (Hashimoto et al., 2012; Honke & Kotani, 2012; Ishiura et al., 2010; Iwamaru et al., 2015; Jiang et al., 2012; Kotani et al., 2008; Miyagawa-Yamaguchi, Kotani, & Honke, 2014, 2015; Yamashita, Kotani, Ishiura, Higashiyama, & Honke, 2011). Fluorescein arylazide reduces the cytosolic background generated by biotin-aryl azide (Jiang et al., 2012), which is membrane permeable during the EMARS reaction and activated by endogenous enzymes (Honke & Kotani, 2012; Kotani et al., 2008); and (b) the selective proteomic proximity labeling assay using tyramide (SPPLAT) method using biotin-tyramide, which is also known as biotin-phenol (Li et al., 2014; Rees, Li, Perrett, Lilley, & Jackson, 2015).

HRP has been used extensively for other applications, such as ELISA and immunochemistry (Ryan, Carolan, & O'Fagain, 2006). Further, antibody-HRP conjugates have been generated that can also be used for proximity labeling. However, this application is limited by the affinity of the antibody. Nevertheless, antibody-HRP conjugates have been successfully used to identify cell surface molecules such as the composition of the B cell receptor cluster, proteins that

interact with Thy1, β1 integrin, CD20, and PrPC, and signaling ligands (Chang et al., 2017; Hashimoto et al., 2012; Honke & Kotani, 2012; Ishiura et al., 2010; Iwamaru et al., 2015; Jiang et al., 2012; Kotani et al., 2008; Li et al., 2014; Miyagawa-Yamaguchi et al., 2014, 2015; Wu et al., 2017; Yamashita et al., 2011). Antibody-HRP conjugates can also be used to identify proteins in fixed cells (Bar et al., 2018).

New versions of HRP have been isolated with modified functions. A bimolecular complementation version of HRP has recently been reported (Martell et al., 2016). This split HRP has been generated to characterize intercellular protein–protein interactions and visualize synapses. The two split HRP fragments were fused with neurexin and neuroligin, which bind to each other across the synaptic cleft. When the split fragments are brought together as a result of the neurexin-neuroligin interaction, they reconstitute a functional form of HRP that allows proximity labeling. This binary system offers another level of control to the HRP system, making it useful for finer spatial restriction. In addition, two enhanced versions of HRP have been isolated. vHRP (Yamagata & Sanes, 2018) was isolated based on stabilizing mutations identified in split-HRP. In parallel, eHRP (Cruz-Lopez, Ramos, Castilloveitia, & Schikorski, 2018) was isolated based on directed evolution. Although split HRP and the enhanced HRP variants have not yet been used for proteomics, their potential use for proteomic mapping of cell–cell interactions is very promising.

5 | APEX-BASED PROXIMITY LABELING

APEX, an engineered ascorbate peroxidase derived from plants, uses the same labeling chemistry and rapid kinetics as HRP to convert a substrate into a radical in the presence of H_2O_2 (Martell et al., 2012; Rhee et al., 2013). The key advantage of APEX over HRP, however, is that it remains active in the reducing environment of the cellular cytosol. Upon activation by H_2O_2, APEX catalyzes the conversion of its substrate biotin-phenol into short-lived (<1 ms) and highly reactive radicals, which can covalently attach to electron-rich amino acids such as tyrosine in nearby endogenous proteins (Hung et al., 2014; Rhee et al., 2013). The labeling reaction can be stopped by the removal of H_2O_2 and the addition of quenching buffer, and the resulting biotinylated proteins can be subsequently isolated using streptavidin beads and further analyzed by MS. In addition, APEX can catalyze the polymerization and precipitation of DAB creating a contrast after OsO_4 fixation (Martell et al., 2012), which can then be used for EM to visualize the structures where APEX is expressed.

Yeast display selection has been performed to screen for mutations that increase APEX activity (Lam et al., 2015). An improved version of APEX, called APEX2, has one additional mutation (A134P) and catalyzes the same chemistry as APEX but with higher activity and sensitivity for promiscuous labeling and EM. APEX2 was further improved with a mutation (C32S) that improved the stability of APEX2-tagged proteins (Huang et al., 2019). Two groups developed a split-APEX2 where inactive fragments of APEX2 can reconstitute and restore enzymatic activity. One group split APEX2 at amino acids 201/202 (Xue et al., 2017), whereas a second group split APEX2 at nearly the same site (200/201) (Han et al., 2019) but used directed evolution of the N-terminal fragment to increase the activity of the reconstituted enzyme.

APEX-mediated proximity labeling was first introduced by Rhee and colleagues to circumvent the limitations of traditional mitochondrial purification and to achieve spatial and temporal specificity of organelle proteome mapping (Rhee et al., 2013). As biotin-phenoxyl radicals are not membrane-permeable, APEX is excellent for proteomic profiling of membrane-enclosed subcellular compartments, such as the mitochondria (Chen et al., 2015; Hung et al., 2014; Rhee et al., 2013) and autophagosomes (Le Guerroue et al., 2017). Nevertheless, APEX is not limited to membrane-enclosed organelles, and has been used successfully to map proteins in the cilia (Kohli et al., 2017; Mick et al., 2015), SGs (Markmiller et al., 2018), mitochondria-ER contact points (Cho et al., 2017; Hung et al., 2017), *Drosophila* ring canals (Mannix, Starble, Kaufman, & Cooley, 2019), mitochondrial nucleoid (Han et al., 2017), bacterial-host inclusion membrane (Olson et al., 2019), lipid droplets (Bersuker et al., 2017), and lysosome-RNA granule contact points (Liao et al., 2019). APEX also provides a good tool for identification of protein–protein interactions. For example, APEX fused with bait proteins have revealed interaction networks of VAPB (James et al., 2019), OPTN (Heo et al., 2019), Rab proteins (Del Olmo et al., 2019), PAQR3 (Cao et al., 2018), MIEF1 microprotein (Rathore et al., 2018), FGF1 (Zhen, Haugsten, Singh, & Wesche, 2018), ribosome-associated quality control complex (Zuzow et al., 2018), and DNA repair factors (Gupta et al., 2018). In particular, the fast labeling time of APEX has been leveraged to identify dynamic changes in protein complex composition (Lobingier et al., 2017; Paek et al., 2017). APEX has also been used for identification of proteins interacting with specific sequences of RNA (Kaewsapsak, Shechner, Mallard, Rinn, & Ting, 2017; Lu & Wei, 2019; Ramanathan et al., 2018) and DNA (Gao et al., 2018; Myers et al., 2018; Qiu et al., 2019). Finally, we note that APEX has recently been used to directly label and identify RNAs (Fazal et al., 2019; Padron, Iwasaki, Ingolia, & Proximity, 2019; Zhou et al., 2019).

6 | PUP-IT PROXIMITY LABELING

Recently, a new proximity labeling system using the bacterial PafA enzyme was developed called PUP-IT (Liu et al., 2018). Unlike BioID, HRP, and APEX, which tag proteins with biotin (known as biotinylation), PafA tags proteins with a small protein called Pup (known as pupylation). In bacteria, PafA ligates Pup to lysine residues on target proteins, signaling those proteins for degradation. During this reaction, Pup is deaminated at its C-terminus to form Pup(E) (also known as Pup_{Glu}), which PafA phosphorylates and conjugates to a lysine residue (Iyer, Burroughs, & Aravind, 2008). PafA has no consensus binding motif flanking the target lysine, and therefore should ligate Pup to any lysine residue in proximity, making it a potentially useful promiscuous protein-labeling enzyme.

To test the effectiveness of PUP-IT as a proximity labeling system, Liu et al. fused PafA to bait proteins and supplied Pup(E) either as purified protein or via transgenic expression and translation into the cell cytoplasm. This resulted in pupylation of proteins in the close vicinity of the enzyme—PafA itself, the bait protein, and interacting prey proteins—but not distant proteins, which indicates a highly specific proximity-dependent labeling reaction. Pupylated proteins can be detected by molecular weight laddering on protein gels or western blots. In addition, the authors devised a more versatile method for detection of pupylation by fusing a bacterial-derived carboxylase domain (BCCP) to Pup(E). BCCP is biotinylated by endogenous ligases in human cells, allowing "bio-Pup(E)" and pupylated proteins to be detected by western blot using streptavidin-HRP, or purified on streptavidin beads and identified by MS. Using this method, the authors identified known interactors on the intracellular tail of CD28 such as p85. Recently, the PUP-IT system was combined with CRISPR-Cas13a (called CRUIS) to identify RNA-binding proteins (Zhang et al., 2020).

Whereas Pup(E) is 64 aa long, two smaller Pup variants were identified called DE28 (28 aa) and Peptide 4.1 (Sun et al., 2020) (14 aa). In particular, Peptide 4.1 lacks lysine residues, which may be useful to prevent unwanted branched tags. While these smaller Pup variants may be useful improvements to the PUP-IT system, they have not been tested under conditions of transgenic expression like Pup(E). Finally, like improvements to BioID, HRP, and APEX, directed evolution of the PafA enzyme may yield increased or modified labeling activity.

7 | COMPARISON BETWEEN BIOTIN LIGASE-BASED, PEROXIDASE-BASED, AND PUP LIGASE-BASED APPROACHES

The major differences between biotin ligase-based, peroxidase-based, and Pup ligase-based (PUP-IT) labeling approaches are the substrates, the targeted amino acid(s), the kinetics, and the working conditions (Figure 2). In addition to differences in proteomic labeling, APEX, like HRP, can be used for EM, thus allowing confirmation of fine subcellular localization. On the other hand, the proper expression and localization of promiscuous biotin ligases and PafA can only be verified by other methods like immunostaining and/or Western blotting to rule out the possibility of false positive from mislocalization of the fusion proteins or slow translation of the fusion protein.

One major difference is the type of substrate used for proteomic analysis. The biotin ligase-based method uses biotin, the peroxidase-based approaches use biotin-phenol, and the PUP-IT method uses biotinylated forms of Pup(E). Delivery of the substrate to the region of interest is a critical factor. Biotin is actively imported into mammalian cells and other organisms though distinct mechanisms (Azhar, Booker, & Polyak, 2015). Even though biotin-phenol can be simply incubated with mammalian cells for cytosolic and mitochondrial protein labeling, a number of studies have shown that biotin-phenol may not effectively penetrate membranes (Li et al., 2014; Rees et al., 2015). Moreover, special procedures are required for efficient delivery of biotin-phenol and optimal proximity labeling in yeast (Hwang & Espenshade, 2016; Singer-Kruger et al., 2020). Therefore, optimizing biotin-phenol delivery to a region of interest in a specific cell type may be required to achieve successful protein labeling. Chemically synthesized bio-DE28 and bio-Peptide4.1 can also be incubated with cells but would likely not penetrate the PM. In contrast, genetically encoded BCCP-PupE is translated into the cytoplasm where it is biotinylated by endogenous ligases. While PupE has the unique advantage of being genetically modifiable with additional domains, this tag is substantially larger than biotin and may interfere with protein function.

The half-life of biotin-5′-AMP radicals generated by promiscuous biotin ligases is on the order of minutes in aqueous solutions (Demoss, Genuth, & Novelli, 1956), which is longer than that of APEX-generated biotin-phenoxyl radicals (<1 ms) (Hung et al., 2014; Rhee et al., 2013). The shorter half-life of unstable radicals may result in a smaller labeling radius, which is also determined by other factors, such as local intracellular environments. Unfortunately, the labeling radius of promiscuous biotin ligases and APEX has been estimated by different methods and in different cellular

regions. Unlike biotin ligase and peroxidase-based approaches, PafA enzyme does not release the Pup tag, thus ensuring that only proteins in close contact with PafA become labeled. Therefore, PUP-IT labeling will likely not be as useful for spatial proteomics such as organelle mapping. Furthermore, the lack of a diffusible reactive substrate may spatially limit labeling to lysine residues on prey proteins that directly face PafA.

Promiscuous biotin ligases and PafA labels lysine residues of nearby proteins whereas APEX and HRP tag electron-rich tyrosine residues. Generally, the estimated amount of lysine present in proteins is higher than that of tyrosine (Echols et al., 2002; Tourasse & Li, 2000). Thus, when the number of available tyrosine residues is limited, potential target proteins may not be identified using APEX and HRP.

Promiscuous biotin ligases and PafA overall show slower kinetics than APEX or HRP. The optimal labeling time for APEX (∼1 min) is shorter than that for HRP (5–10 min) and much shorter than for BioID (15–24 hr) and PafA (24 hr). The only exception is TurboID and miniTurbo, which label on timescales closer to APEX and HRP (∼10 min). Although biotin is not toxic, biotinylation of proteins over a long period may perturb protein function, lead to artificial interactions, and cause cell toxicity, which was confirmed in cultured mammalian cells expressing TurboID longer than 24 hr (Branon et al., 2018). This difference in labeling time will undoubtedly change the specificity of the labeled proteomes. While promiscuous biotin ligases and PUP-IT are useful for capturing entire changes in protein complexes during a longer period of time, APEX is excellent for characterizing rapid dynamic changes in proteomes that can only be achieved with a short labeling window, such as acute responses to drug treatment (Lobingier et al., 2017; Paek et al., 2017). The fast labeling times of TurboID suggests it too can be applied in this manner.

Notably, the activity of BioID or BioID2 is greatly reduced at temperatures below 37°C[13]. For model systems that need to be maintained under 37°C, BioID cannot be easily used. Nevertheless, BioID has been successfully applied to many organisms in addition to mammalian cells, such as single celled organisms (*Trypanosoma brucei*, *T. gondii*, *Dictyostelium discoideum*, *Plasmodium berghei*), invertebrates (*Drosophila melanogaster, Caenorhabditis elegans*), and plants (*Nicotiana benthamiana*, *Arabidopsis thaliana*) (Batsios, Ren, Baumann, Larochelle, & Graf, 2016; Chen et al., 2015; Dingar et al., 2015; Hu, Zhou, & Li, 2015; Kehrer, Frischknecht, & Mair, 2016; McAllaster et al., 2015; Morriswood et al., 2013; Zhou, Hu, & Li, 2016). In contrast, TurboID and miniTurbo were evolved in yeast grown at 30°C, perhaps explaining why they perform well in *Drosophila* and *C. elegans*, which are grown at 25 and 20°C, respectively. APEX has been shown to be active in *Drosophila* cultured cells at 25°C and in yeast cultured at room temperature, in addition to showing good activity in mammalian cells that are cultured at 37°C. This temperature range allows APEX to be broadly suitable for studies in a variety of model organisms.

8 | COMPARISON BETWEEN APEX AND HRP-BASED APPROACHES

Both APEX and HRP catalyze the same proximity labeling chemistry. The key parameter that one should consider for their usage is the environment to which the enzyme will be exposed. As mentioned above, HRP is inactive in the cytosol; however, it is functional when it faces outside the cell on the cell surface and has been successfully used to identify membrane proteins (Bausch-Fluck, Milani, & Wollscheid, 2019; Chang et al., 2017; Cijsouw et al., 2018; Hashimoto et al., 2012; Honke & Kotani, 2012; Ishiura et al., 2010; Iwamaru et al., 2015; Jiang et al., 2012; Kotani et al., 2008; Li et al., 2014, 2020; Loh et al., 2016; Miyagawa-Yamaguchi et al., 2014, 2015; Wu et al., 2017; Yamashita et al., 2011). Notably, many previous studies used antibody-conjugated HRP (Bar et al., 2018; Chang et al., 2017; Hashimoto et al., 2012; Honke & Kotani, 2012; Ishiura et al., 2010; Iwamaru et al., 2015; Jiang et al., 2012; Kotani et al., 2008; Li et al., 2014; Miyagawa-Yamaguchi et al., 2014, 2015; Wu et al., 2017; Yamashita et al., 2011). A key advantage of the HRP-mediated approach is that many antibody-HRP conjugates are currently available. As noted previously, however, the use of antibody-conjugated HRP in proximity labeling is limited by the affinity of the antibody.

9 | ANALYSIS OF PROTEOMIC DATA FROM PROXIMITY LABELING APPROACHES

A challenge common to all labeling strategies is to distinguish candidate proteins from background in MS data. Generally, proteins with the highest abundance, and represented by 2 or more independent peptides, are chosen for further study even though low-abundance candidates may potentially be biologically relevant. Researchers have devised additional experimental procedures to help generate a high-confidence and comprehensive list of candidates from MS data:

(a) Proximity labeling coupled with quantitative MS can be achieved using metabolic labeling such as SILAC (stable isotope labeling by amino acid in cell culture) (Ong et al., 2002) or done with in vitro chemical labeling, such as iTRAQ (isobaric tags for relative and absolute quantification) (Ross et al., 2004) and TMT (tandem mass tags) (Thompson et al., 2003). (b) Additional negative controls can help filter out background labeled proteins. For example, researchers can target a labeling enzyme to a different organelle or protein complex in addition to the primary target (Hung et al., 2014). Furthermore, isogenic cell lines can be used to avoid differences in transgene expression (Hesketh, Youn, Samavarchi-Tehrani, Raught, & Gingras, 2017; Vandemoortele et al., 2019). (c) Background due to nonspecific labeling can be reduced by inducibly activating the labeling enzyme (Cho et al., 2017; Kehrer et al., 2016) or using endogenous CRISPR/Cas9 tagging of bait proteins to maintain physiological levels of the labeling enzyme (Long, Brown, & Sibley, 2018). (d) True positives can be distinguished by identifying peptide biotinylation sites (Kim et al., 2018; Lee et al., 2016; Udeshi et al., 2017). See additional reviews for detailed considerations for proximity labeling experimental design and data analysis (Gingras, Abe, & Raught, 2019; Samavarchi-Tehrani, Samson, & Gingras, 2020).

10 | PROXIMITY LABELING IN DEVELOPMENTAL SYSTEMS

Proximity labeling is typically performed in cultured cells due to technical advantages of this system (e.g., easy delivery of labeling reagents, efficient cell lysis of large quantities of cells). However, the application of proximity labeling tools in vivo has specific benefits. For example, in vivo protein labeling allows researchers to identify organelle components or protein interactions from cells in a normal physiological environment, including cell types that would be too difficult grow in culture (e.g., neurons (Han, Li, & Ting, 2018)). Furthermore, by expressing labeling enzymes from transgenes, protein labeling can be restricted to specific cell types or developmental stages. Cells expressing labeling enzymes can also be transplanted into otherwise wild-type host organisms.

Penetration of labeling substrate into target tissues and cells is a significant technical challenge of using proximity labeling tools in vivo. For example, experiments using APEX or HRP require incubating live dissected tissues with biotin-phenol. For some experiments, this dissection step might be too laborious, or make it difficult to collect enough material for pulldown/MS analysis. In contrast, promiscuous biotin ligases can label proteins in intact organisms. This is because biotin is membrane permeable and can be added to an organism's water/food supply. Temporal labeling experiments may be difficult using this method, as biotin needs to ingested and perfuse to the target tissue. For example, *Drosophila* adult flies expressing TurboID exhibit significant labeling only after 16 hr of feeding flies biotin (Branon et al., 2018). This problem might be addressed by direct injection of biotin into the organism (Han et al., 2018), or temporal control of biotin ligase expression. Finally, while Pup-IT has yet to be applied in vivo, the PupE label can be genetically encoded, potentially avoiding tissue penetration entirely.

Many groups have applied proximity labeling tools in developmental systems, such as *Arabidopsis* (Khan, Youn, Gingras, Subramaniam, & Desveaux, 2018; Kim et al., 2019; Mair et al., 2019), *C. elegans* (Branon et al., 2018; Reinke, Mak, Troemel, & Bennett, 2017), *Drosophila* (Branon et al., 2018; Chen, Hu, et al., 2015; Li et al., 2020; Mannix et al., 2019; Shinoda, Hanawa, Chihara, Koto, & Miura, 2019), and mouse (Brudvig et al., 2018; Dingar et al., 2015; Uezu et al., 2016). Importantly, some have used proximity-labeling tools to discover new components of developmental processes. For example, APEX was used in *Drosophila* to identify novel components of the ring canals, which are intercellular pores that transport cellular material from nurse cells to the developing oocyte (Mannix et al., 2019). By tagging known ring canal proteins with APEX, and phenotypic screening MS hits by RNAi, they identified eight new proteins important for ring canal morphology. Another study in *Drosophila* used HRP localized to the cell surface to identify new wiring regulators in developing and adult olfactory projection neurons (Li et al., 2020). RNAi screening of MS hits revealed 20 new developmental regulators of olfactory projection neuron wiring, including the lipoprotein receptor LRP1. Finally, in *C. elegans*, APEX was expressed under the control of four different tissue-specific enhancer elements, as well as targeted to either the nucleus or cytoplasm (Reinke et al., 2017). By comparing MS datasets from each condition, they identified tissue specific and subcellular specific proteins, seven of which were confirmed by visualizing proteins tagged with green fluorescent protein (GFP) and had no previous such annotation.

11 | CONCLUSION/PERSPECTIVES

Since the recent introduction of proximity labeling, the method has made significant contributions to the mapping of local interactomes relevant to a wide range of biological processes. By tagging regional proteomes, proximity labeling

overcomes issues associated with traditional approaches of organelle purification and allows proteomic analysis of other types of subcellular regions. A disadvantage that all proximity labeling-based methods have in common is that they cannot distinguish direct binding of two proteins from proximity of two adjacent proteins. Thus, these methods serve as discovery methods that require detailed follow-up studies. Nevertheless, as proximity labeling does not require disruption of cells for complex isolation, these methods not only preserve evidence of weak or transient interactions that are not detectable using traditional approaches but also minimizes false discovery by eliminating false positives generated during lysis or disruption. Importantly, as proximity labeling can be performed in living cells, researchers can study protein–protein interactions and proteomic alterations in physiologically-relevant conditions. Proximity labeling has been adapted to several model systems, making this technology available to study diverse biological problems in a wide range of organisms.

Notably, while improved variants of labeling systems are now available (Figure 3), further improvements are likely to be made in the near future. In particular, variants of PafA with faster kinetics and higher activity could be isolated that match the robustness of APEX2 and TurboID enzymes. Furthermore, PafA variants that release diffusible reactive PupE, similar to promiscuous biotin ligase-based and peroxidase-based systems, would make the PUP-IT system more useful for spatial proteomics such as organelle mapping. Furthermore, a split-PafA enzyme would be a valuable addition to existing split labeling tools to fine-tune spatial restrictions.

Importantly, the ease of applying genetically encoded enzymes will benefit greatly from the powerful genome editing using CRISPR technology (Housden & Perrimon, 2016; Komor, Badran, & Liu, 2016), as these enzymes can now be easily fused to any gene of interest via a knock-in approach. In addition, numerous genetic engineering tools already available for organisms such as *Drosophila* facilitate a wide range of proximity-labeling applications. For example, the existing library of MiMICs, a transposon insertion resource for engineering *Drosophila* genes, allows for rapid tagging of genes (Nagarkar-Jaiswal et al., 2015; Venken et al., 2011). Altogether, a broad-range of proximity-labeling applications that build on existing tools are now possible and likely to provide deep insights into various biological questions.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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