Proximity-dependent biotinylation approaches to study apicomplexan biology

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Abstract
In the last 10 years, proximity-dependent biotinylation (PDB) techniques greatly expanded the ability to study protein environments in the living cell that range from specific protein complexes to entire compartments. This is achieved by using enzymes such as BirA* and APEX that are fused to proteins of interest and biotinylate proteins in their proximity. PDB techniques are now also increasingly used in apicomplexan parasites. In this review, we first give an overview of the main PDB approaches and how they compare with other techniques that address similar questions. PDB is particularly valuable to detect weak or transient protein associations under physiological conditions and to study cellular structures that are difficult to purify or have a poorly understood protein composition. We also highlight new developments such as novel smaller or faster-acting enzyme variants and conditional PDB approaches, providing improvements in both temporal and spatial resolution which may offer broader application possibilities useful in apicomplexan research. In the second part, we review work using PDB techniques in apicomplexan parasites and how this expanded our knowledge about these medically important parasites.

KEYWORDS
apicomplexa, APEX, BioID, BirA, malaria, Plasmodium, protein-protein interaction, proximity-dependent biotinylation, Toxoplasma

1 | PRINCIPLES AND MODIFICATIONS OF PDB

1.1 | Principle of proximity-dependent biotin labeling

Understanding protein–protein interactions are essential for gaining mechanistic insights into most biological processes. Proximity-dependent biotinylation (PDB) is used as an interactome discovery method in living cells that involves the covalent modification of proteins with biotin based on their proximity to a protein of interest (Rees et al., 2015b; Roux, 2013; Varnaitė & MacNeill, 2016). PDB uses engineered enzymes that catalyze the biotinylation of proteins upon activation by the substrate (biotin or derivatives; Figure 1a,b). To achieve this in living cells, the enzyme is directly fused to the protein of interest (the bait) for which interactors (the prey) are searched. Alternatively, if proteins from a particular cellular compartment are to be identified, the enzyme is fused with a trafficking signal to target it to the cellular area or compartment of interest. The biotinylated proteins can be specifically enriched via standard pull-down methods using streptavidin-coated beads and subsequently identified by mass spectrometry (MS).

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The key enzymes for PDB are **biotin ligases** and **peroxidases** (Table 1). Although both enzyme classes result in biotinylated proteins, they use different chemical reactions (Figure 1a), leading to different labeling properties (reviewed in detail in Samavarchi-Tehrani et al., 2020). The Escherichia coli biotin ligase BirA catalyzes the formation of an amide bond between reactive biotin and a lysine residue (Figure 1a). Biotinylation occurs in a highly site-specific fashion, a property originally exploited to investigate known interaction partners by fusing one partner with BirA and the other with a short lysine-containing peptide tag (Beckett et al., 1999; Cronan, 1990; Fairhead & Howarth, 2015). Since this approach is only suitable to biotinylate known targets (Fernández-Suárez et al., 2008; Roux, 2013), the active site of BirA was mutated (R118G) to achieve a promiscuous version of the biotin ligase (BirA*; Kwon et al., 2000). BirA* indiscriminately biotinylates primary amines of proximate proteins (i.e., lysine residues or protein N-termini; Choi-Rhee et al., 2004; Kwon & Beckett, 2000; Kwon et al., 2000) and was exploited for the first PDB technique known as proximity-dependent biotin identification (BioID; Roux et al., 2012). BirA*-based labeling acts through the formation of a biotin-adenylate ester (biotinoyl-5'-AMP, Figure 1a) with a half-life of minutes. Optimal biotinylation of proximate proteins requires a labeling period of 15–18 hr (Kim et al., 2014; Roux et al., 2012; Figure 1b) during which BirA* captures protein associations and accumulates a history of protein interactions over a time period. An effective labeling radius of ~10 nm was reported for BioID in a study of the nuclear pore complex (Kim et al., 2014; Figure 1b).

Peroxidases generally catalyze redox reactions. In the context of PDB, peroxidases generate phenoxyl radicals that covalently react with electron-rich side chains of amino acids such as tryptophan or tyrosine by oxidizing biotinylated substrates while reducing the cosubstrate hydrogen peroxide (Bhaskar et al., 2003; Gross & Sizer, 1959; Minamihata et al., 2011; Schönhuber et al., 1997; Figure 1a). The horseradish peroxidase C (HRP) has been used for PDB in approaches such as enzyme-mediated activation of radical source (EMARS; Honke & Kotani, 2011; Kotani et al., 2008) and selective proteomic proximity labeling assay using tyramide (SPPLAT; Figure 1b; Li et al., 2014; Rees et al., 2015a). These approaches established either biotin-labeled aryl azide or a biotinylated tyramine derivative (biotin phenol) as a labeling reagent, respectively. Biotin phenol is also exploited for PDB with an engineered plant ascorbate peroxidase (APEX; Rhee et al., 2013) that was initially developed as an enzyme to detect the location of proteins of interest by electron microscopy (Martell et al., 2012). Due to its poor activity and the oxidative damage by hydrogen peroxide of the original APEX enzyme (Rhee et al., 2013), PDB approaches mainly use the improved variant APEX2 (Lam et al., 2015). The cells expressing peroxidases are first incubated with biotin phenol and subsequently treated with hydrogen peroxide to initiate the labeling reaction (Figure 1b). Since peroxidases have a higher enzymatic reactivity compared with biotin ligases and generate short-lived biotin-phenoxyl radicals, the labeling can be performed for as little as 1 min before quenching the reaction, leading to a time-restricted snapshot of protein proximity (Kim & Roux, 2016). Peroxidases are therefore ideal to study short-lived events, whereas BioID is well suited to provide the interaction history over a longer time period. In contrast to BirA*, peroxidases biotinylate proximate proteins within a wider labeling radius ranging between 20 and 300 nm (depending on the method; Figure 1b; Kotani et al., 2008; Rhee et al., 2013). However, as peroxidases rely on the less frequently surface-exposed aromatic amino acids (Minamihata et al., 2011), the labeling may be less frequent and labeled proteins appear to be more difficult to detect compared to PDB with BirA* which labels the more abundant lysine residues (Cronan & Reed, 2000; Rees et al., 2015b).

### 1.2 General properties of proximity-dependent biotin labeling

A particular strength of PDB is the labeling of proteins in living cells while molecular complexes and cellular compartments stay intact, thereby preserving the spatial association and interaction network in their natural environment. Although BirA* suffers from a reduced activity below 37°C (Kim et al., 2016), APEX has been shown to be active at a broad temperature range (20–37°C; Chen, Hu, et al., 2015; Hwang & Espenshade, 2016) and thus is suitable for a variety of organisms, including, for instance, apicomplexan insect parasites that are kept at temperatures below 30°C (see Section 3). However, even with a short labeling period (and improved enzyme versions like APEX2), there is a risk of potential oxidative stress from the use of highly cytotoxic hydrogen peroxide that can make peroxidases challenging to apply in living organisms. Although biotin itself is not toxic, high biotin concentrations and the biotinylation of proteins over the long BioID labeling time may also impact cellular functions.

There are two nonmutually exclusive theoretical outcomes of a PDB experiment: labeling of proteins in a complex with the bait or labeling of the entire cellular substructure or compartment where the bait is located in (Figure 1c). The labeling density is highest at the site of the bait and decreases in a distance-dependent manner (Hung et al., 2016; Kim et al., 2014). This favors the labeling of interactors and proteins found in a permanent close spatial location with respect to the bait. However, as a physical interaction of the bait is not needed for labeling, general labeling of the proteins of the same cellular compartment occurs. This is most pronounced if the labeling area is fenced with membrane boundaries because the biotin ligase and peroxidase-generated reactive species are not membrane permeable (Rhee et al., 2013). Depending on the chosen PDB enzyme, the balance between these two principle outcomes of either protein complex interactomes or proteomes from subcellular compartments varies (Figure 1c). Due to the comparably small labeling footprint, biotin ligases are best suited to discover the spatial distribution of proteins within a protein complex and associated proteins in the immediate vicinity. This was first demonstrated with BirA* directly fused to the bait to identify constituents (interacting and neighboring proteins) of the nuclear lamina (Kim et al., 2014; Roux et al., 2012). In contrast, peroxidases excel at compartment proteomics due to their large labeling footprint. To selectively label as many of the compartment-specific proteins as possible, the enzyme is targeted to the organelle...
**FIGURE 1** PDB principles and applications. (a) Biotin ligases and peroxidases catalyze the addition of biotin (yellow) to preferred residues (purple and orange) through different chemical reactions. Related substrates result in different intermediate products. (b) Scheme of PDB enzyme labeling properties inside a cell or at the cell surface. The enzymes are utilized as HRP-coupled antibodies (EMARS/SPPLAT), separately expressed in the cell (here shown with APEX) or directly fused to the bait (here shown with BioID). Incubation (typical labeling times are indicated) with specific substrates leads to the biotinylation of residues of proteins proximal to the bait (labeling radius indicated). (c) Two principle outcomes of PDB experiments are favored by either APEX or BioID, respectively. (d) Protein structure schematics of PDB enzyme variants. Biotin ligases were made promiscuous by introducing a mutation (indicated as a bold green line) in the catalytic region. Improved enzyme versions contain additional mutations (indicated as fine green lines). For split PDB enzyme versions, the enzyme is divided at the indicated split site. (e) Working principle of adapted PDB approaches.
| Type            | Enzyme          | Origin            | Size | Mutations | Labeling | Applications | Apicomplexa/stages | References                              |
|-----------------|-----------------|-------------------|------|-----------|----------|--------------|-------------------|-----------------------------------------|
| Biotin ligase   | BirA*           | *Escherichia coli*| 35   | R118G     | 18 hr    | IMC          | Toxoplasma gondii tachyzoites | Chen Kim et al. (2015), Chen et al. (2017) |
|                 |                 |                   |      |           |          | Conoid       | *T. gondii* tachyzoites          | Gaji et al. (2015)                        |
|                 |                 |                   |      |           |          | Dense granules Conoid | *T. gondii* tachyzoites          | Nadipuram et al. (2016)                  |
|                 |                 |                   |      |           |          | Mitochondria | *T. gondii* tachyzoites          | Long Anthony et al. (2017), Long Brown et al. (2017) |
|                 |                 |                   |      |           |          | Dense granules Conoid | *T. gondii* tachyzoites          | Seidi et al. (2018)                      |
|                 |                 |                   |      |           |          | Cyst wall    | *T. gondii* tachyzoites          | Pan et al. (2019)                       |
|                 |                 |                   |      |           |          | Dense granules | *T. gondii* bradyzoites      | Koreny et al. (2021)                    |
|                 |                 |                   |      |           |          | Microtubule binding proteins | *T. gondii* bradyzoites      | Tu et al. (2020)                        |
|                 | Biotin ligase   | *Escherichia coli*| 27   | R40G      | 18 hr    | IMC          | *T. gondii* tachyzoites          | Engelberg et al. (2020)                 |
|                 | BASU            | *Bacillus subtilis*| 28   | N-terminal deletion: R142G, E323S, G323R | 18 hr    | n/a          | *T. gondii* tachyzoites          | Boucher et al. (2018)                    |
|                 | TurboID         | *Escherichia coli*| 35   | 14 mutations including R118S | 5-10 min | n/a          | *T. gondii* tachyzoites          | Khosh-Naucke et al. (2018)              |
|                 | miniTurbo       | *Escherichia coli*| 28   | N-terminal deletion: 12 mutations including R118S | 5-10 min | PVM          | *T. gondii* tachyzoites          | Schnider et al. (2018)                  |
|                 | AirID           | Ancestral         | 37   | R118S     | 3 hr     | n/a          | *T. gondii* tachyzoites          | Birnbaum et al. (2020)                  |
|                 | Peroxidase      | Horseradish       | 44   |           | 5-10 min | n/a          | *T. gondii* tachyzoites          | Geiger et al. (2020)                    |
|                 | APEX            | Pea               | 28   | K14G, E112K, W41F | 1 min   | Mitochondria | *T. gondii* tachyzoites          | Wichers et al. (2021)                   |
|                 | APEX2           | Soybean           | 28   | K14G, E112K, W41F, A134P | 1 min   | Dense granules Micronemes Nucleus and cytoplasm | *T. gondii* tachyzoites          | Kehrer et al. (2016)                    |
|                 | Pup-ligase      | PUP-IT            | 54   | Q64E      | 24 hr    | n/a          | *P. berghei* gametocytes         | Kehrer et al. (2020)                    |
|                 | SrtA            | *Staphylococcus aureus* | 35   |           | 1 hr     | n/a          | T. gondii* host cells           | Rosenberg and Sibley (2021)             |

Abbreviations: n/a, no application to date; size in kDa; SrtA, transpeptidase sortase A.
of interest (Figure 1b), as originally demonstrated with APEX targeted to the mitochondrial matrix (Rhee et al., 2013). Of course, APEX is also applicable to analyze protein interactions if the enzyme is fused to a specific target protein (Hung et al., 2014; Rhee et al., 2013) and, vice versa, BioID can be used to map the proteome of subcellular structures (Gupta et al., 2015). In contrast to APEX, which is usually fused to a targeting signal for the compartment of interest, HRP is typically conjugated to antibodies directed against the target structure such as the cell surface of living cells, and rarely expressed as a fusion protein (Figure 1b; Kotani et al., 2008; Miyagawa-Yamaguchi et al., 2014). Unlike APEX that can function intracellularly (Rhee et al., 2013), HRP is inactive in reducing environments such as the cell cytosol but retains its catalytic activity in oxidizing environments such as the cell surface or structures associated with the secretory pathway (Hopkins et al., 2000; Martell et al., 2012; Samavarchi-Tehrani et al., 2020). Besides proximity labeling, HRP and APEX can create electron-dense contrast to visualize the location of proteins in the cell using electron microscopy (Ellisman et al., 2012; Martell et al., 2012). This has already been applied in malaria parasites (Charnaud et al., 2018).

Typically, a PDB experiment results in large numbers of MS protein hits. The identified candidates may either be direct compartment neighbors or proteins in a fixed spatial arrangement of the bait but also false positives that randomly come into proximity of the bait due to molecular crowding. Therefore, a fundamental requirement is an appropriate experimental design, the implementation of controls, and multiple experimental runs (see Hung et al., 2016; Kim & Roux, 2016; Roux et al., 2018; Sears et al., 2019 for experimental set-up guides). In addition, and similarly to other interactome methods such as co-immunoprecipitations, it is essential that the identified candidates are verified to be interactors by independent methods (see Section 1.4 for some options to validate PDB hits) or to confirm their location in the target compartment.

1.3 | Improvements and adaptations of PDB methods

Since their initial development and application, a growing number of novel methods have emerged providing improvements in terms of sensitivity, speed, and size of the PDB enzyme and moreover, adapted the original PDB approaches to offer further application possibilities (Tables 1 and 2). Although at their core, these approaches use similar labeling chemistry for protein identification, they can provide an alternative set (either more restricted or broader) of proximal interactors or organelle proteomes. Here we list some promising developments and modifications.

1.3.1 | Improvements

To improve targeting of BioID fusion proteins and reduce the functional interference associated with tagging the bait, a substantially smaller biotin ligase (one of the smallest biotin ligase to date) from *Aquifex aeolicus* was made promiscuous by introducing the R40G mutation that is orthologous to the R118G mutation in BirA* and was termed BiolD2 (Figure 1d; Kim et al., 2016; Tron et al., 2009). BiolD2 also displays a higher affinity for biotin compared with BirA* which allows the use of lower biotin concentrations for protein labeling (the biotin present in the culture medium of some cells suffices). This can be useful for PDB experiments in systems in which biotin supplementation may not be easily accomplished (Kim et al., 2016; Kim and Roux, 2016). However, it also increases the unspecific labeling of highly abundant proteins and limits the control of the labeling extent and timing by adjusting the exogenous biotin concentration in the culture medium. An alternative promiscuous biotin ligase, BASU from *Bacillus subtilis*, was also generated by engineering in the orthologous mutation of R124G in BirA* among additional C-terminal mutations (Figure 1d; Ramanathan et al., 2018). However, these improved biotin ligases still have slow enzyme activity requiring long labeling times that limit the investigation of dynamic processes that can be investigated. To overcome the slow labeling kinetics, a new pair of biotin ligase variants, named TurboID and miniTurbo, was developed using yeast-display-directed evolution (Figure 1d; Branon et al., 2018). Due to the increased enzyme activity, labeling times as short as 5–10 min were reported for sufficient labeling in PDB experiments with these variants (Branon et al., 2018). TurboID and miniTurbo therefore permit probing of dynamic processes with much higher temporal resolution—nearly comparable with peroxidases—while still maintaining the nontoxic labeling conditions characteristic for BioID. However, the high enzymatic activity of TurboID may increase background labeling compared with original BioID (May et al., 2020). TurboID and miniTurbo retain their high activity at lower temperatures, alleviating a further disadvantage of BirA* compared with peroxidase-based approaches that are relevant to nonvertebrate stages of Apicomplexans. Compared with TurboID, miniTurbo is smaller (due to an N-terminal deletion) and retains the lower biotin affinity of BirA*. An ancestral enzyme reconstruction algorithm combined with site-directed mutagenesis gave rise to a further biotin ligase containing the R118S mutation, named ancestral BirA for proximity-dependent biotin identification (AirID; Kido et al., 2020; Figure 1d). In contrast to TurboID that can cause nonspecific biotinylation and cell toxicity under extreme conditions (long labeling times and high biotin concentrations), AirID tolerates a broad range of different conditions and is less toxic for cells over time. Therefore, it can be a robust alternative for long-term experiments in living cells. Most recently, the to-date smallest biotin ligase variants for PDB (molecular weight below 20 kDa), named microID and ultraID, have been developed as truncation variants of BiolD2 (Zhao et al., 2021). UltraID was reported to be active at low concentrations of biotin (similar to BioID2) and very short labeling times were required (similar to TurboID). It will be interesting to see the performance of these newest variants in future work. Of all the available BirA* variants only a very limited number have so far been used in apicomplexans (Section 2).

Since the introduction of APEX2, the only further improvement of this enzyme for PDB was a cysteine-free single mutant that increased
the stability of APEX2-tagged proteins (Huang et al., 2019). Also new versions of HRP have been engineered with modified functions. Based on directed evolution, enhanced HRP (eHRP) was generated to increase the catalytic activity of HRP (Cruz-Lopez et al., 2018). In parallel, a further enhanced variant HRP (vHRP) was developed by introducing mutations that improve the stability of the enzyme (Yamagata & Sanes, 2018). Site-directed mutagenesis yielded a recombinant HRP variant with improved thermal stability and catalytic activity (Humer & Spadiut, 2019). However, these HRP variants have not been used for proteomics so far.

1.3.2 | Adoptions

To reduce the background in PDB experiments, induced dimerization PDB systems were developed (Figure 1e). Two conceptually similar systems, two-component BioID (2C-BioID; Chojnowski et al., 2018), and dimerization-induced quantitative BioID (DiQ-BioID; Birnbaum et al., 2020) take advantage of the well-established FKBP-FRB dimerization system (Putyrski & Schultz, 2012). In contrast to the conventional BioID, one domain of the dimerization system is fused to the bait and separately expressed in the cell with BirA* fused to the other domain. The two components are only brought together following FKBP-FRB dimerization induced by a rapamycin derivative, thereby recruiting the BioID fusion to the target protein (Figure 1e).

Background labeling from freely diffusing BirA* in the cell can be subtracted from the specific labeling of possible interaction partners of the bait and therefore these systems provide a prediction of protein association with high specificity. In addition to BirA*, APEX2 was also exploited for 2C-BioID to further increase the spatial resolution of PDB labeling (James et al., 2019). Of these systems, DiQ-BioID was developed and already successfully applied in apicomplexan parasites (see Section 2).

PDB enzymes have also been adapted to the protein fragment complementation assay (PCA) system that was originally exploited to analyze binary protein interaction using colorimetric or fluorescent reporter molecules (Remy & Michnick, 2004; Shekhawat & Ghosh, 2011). The PDB enzyme is split into two inactive fragments (at defined split sites, Figure 1d) that are simultaneously expressed in the cell, each fused to one of the two potential protein interaction partners (Figure 1e). If the interaction occurs, the biotinylation activity of the split-PDB enzyme is restored and proximal proteins associated with these interacting proteins will be labeled (Figure 1e). Besides the validation of binary protein interaction, split-PDB enzymes can be used to discover interactomes of two proteins that are part of the same protein complex or interactomes which form only under specific conditions, for example, after phosphorylation of one of the target proteins or at membrane contact sites of two different compartments. Split forms of each PDB enzyme that have been created include split-BioID (Munter et al., 2017; Kwak et al., 2020; Schopp et al., 2017), split-TurboID (Cho et al., 2020), split-APEX2 (Han et al., 2019; Xue et al., 2017), and split-HRP (Martell et al., 2016). As with the conventional enzymes, split-HRP is restricted to extracellular application and has not been combined with MS to analyze proximal proteins so far (Samavarchi-Tehrani et al., 2020). Split-BioID systems suffer from the already slow labeling kinetics of BirA* which is lowered even further in the reconstituted enzymes compared with the full-length protein. This problem is mitigated by split-TurboID, which requires shorter labeling times than split-BioID and exhibits more robust labeling after reconstitution (Cho et al., 2020).

Recent work modified PDB as a tool to detect proteins recruited to nucleic acids in living cells (comprehensively reviewed in Qin et al., 2021). The PDB enzyme was fused with inactive Cas13 (Han et al., 2020; Zhang et al., 2020) or Cas9 (Gao et al., 2018; Myers et al., 2018) that enable biotinylation of proteins associated with a target RNA or DNA sequence, respectively. Vice versa, approaches such as APEX-seq (Fazal et al., 2019) and ALaP (Kurihara et al., 2020) enable the enrichment of nucleic acids in specific subcellular locations or that interact with the bait. Another technique that deserves attention is ChromID, where BASU allows the identification of proteins associated with specific chromatin modifications (Villaseñor et al., 2020). However, none of these approaches have been applied to apicomplexan parasites so far.

1.3.3 | New PDB enzymes

As an alternative to biotin ligases and peroxidases as PDB enzymes, pupylation-based interaction tagging (PUP-IT) was developed for proximity labeling of membrane proteins (Liu, Zheng, et al., 2018). In this approach, interacting proteins of the PafA enzyme-fused bait are labeled with a small protein called Pup. In addition to the bait, the pupylation reagent Pup(E) can be directly expressed in cells which makes the addition of a compound (such as biotin) unnecessary. Since the pupylated proteins with the initial substrate Pup(E) can only be detected on immunoblots, the pupylation was coupled with biotinylation of Pup(E) by endogenous biotin ligases for isolation and identification of biotinylated and pupylated proteins using MS. In contrast to BioID and APEX for which the activated substrate freely diffuses to label proximal proteins, in PUP-IT the "biotin-Pup(E)" remains bound to the PafA enzyme, leading to a more restricted labeling radius. This property might be advantageous for investigations of direct protein interactions but may be less useful for organelle mapping and investigation of larger protein complexes (reviewed in Bosch et al., 2021; Santin, 2019; Sears et al., 2019; Trinkle-Mulcahy, 2019). To date, PUP-IT has not been used in apicomplexan parasites.

Another alternative to the key PDB enzymes is the enzyme-mediated proximity cell labeling (EXCELL) to investigate cell surface–surface interactions (Ge et al., 2019). To achieve promiscuous labeling of various cell surface proteins via EXCELL, a promiscuous Staphylococcus aureus transpeptidase sortase A variant (mgSrtA) was developed that is displayed on the surface of the cell of interest. After the addition of a small peptide (LPXTG penta-peptide coupled with biotin) as the substrate, mgSrtA catalyzes the biotinylation of proteins containing a monoglycine residue at the N-terminus. In
contrast to BirA* and APEX-based PDB (see Section 2), the applicability of EXCELL and PUP-IT for apicomplexan parasites remains unknown to date. One possible application of EXCELL in apicomplexan parasites could be to find surface interacting proteins of extracellular life cycle stages (ookinetes and sporozoites).

1.4 Comparison of PDB with other methods to generate interactomes

PDB methods have emerged as powerful tools to determine interactomes or compartment proteomes. However, there are many alternative approaches to achieve similar goals, each with its advantages and limitations. These alternatives include biochemical methods (such as affinity purification or subcellular fractionation) and genetic techniques (such as yeast two-hybrid). We here compare the properties of PDB with those of some common alternative methods.

The main limitation of PDB approaches is the requirement for the expression of the PDB enzyme in the studied cells. First, this requires the capacity for genetic manipulation of the target organism, a requirement fulfilled for the best-studied apicomplexan parasites. Secondly, for approaches in which the PDB enzyme is directly fused to the bait, this can interfere with the function of the bait and directly or indirectly alter its interactions and location in the cell (Stadler et al., 2013; Weill et al., 2019). In addition, it is important to consider the spatial configuration required for successful labeling. Due to an incorrect orientation of proteins to each other or placement of the PDB enzyme, the possibility exists that no labeling of interacting proteins would occur, thus producing false-negative results (Rees et al., 2015b; Roux, 2013). It is therefore essential to ascertain the correct subcellular localization and topology as well as the functional properties of the PDB enzyme-bait fusion.

Affinity purification followed by mass spectrometry (AP-MS) is the most frequently applied method for the discovery of interactomes or spatial proteomics besides PDB (Dunham et al., 2012; Lundberg & Borner, 2019). For approaches such as coimmunoprecipitation (Co-IP), antibodies specific for the bait (or a tag on the bait) are used to isolate protein complexes. Although specific antibodies avoid the need for genetic modification of the bait (e.g., if the organism is not or only poorly genetically tractable as is the case for some less well-studied apicomplexans) and are also very useful if modification of the bait alters critical properties, they are not always available, or their binding properties may be unsuitable for IP. The main disadvantage compared to PDB is that AP-MS approaches suffer a loss of interactors during cell lysis and wash steps prior to detection. This problem frequently occurs when dealing with proteins that make weak and transient interactions, especially when they are of low abundance or if the bait is present in insoluble complexes that may not solubilize in buffers suitable for IP. As a solution for weak or transient interactions, AP-MS can be coupled with crosslinking to curb the reduction of bound interactors during the solubilization of protein complexes. However, this can promote the occurrence of false positives due to artifacts such as aggregation and reduces simplicity of use as crosslinking conditions must be adjusted for each specific type of protein (Dunham et al., 2012).

In contrast to AP-MS which relies on antibody affinity capture that demands certain binding conditions, PDB uses biotinylation which can be purified using streptavidin support which can withstand extremely stringent wash conditions (Roux et al., 2012). Therefore, PDB permits isolation of the prey with high purity which improves the identification of low abundant proteins. Since the key step of biotinylation occurs before the subsequent solubilization, this also eliminates the need to preserve interaction during sample preparation. The independence of retaining interactions during cell lysis and purification also enables the use of buffer conditions that make the prey proteins less prone to degradation. This property is particularly useful for the detection of proteins from cytoskeletal structures (in Apicomplexans, e.g., the IMC, conoid, apical anuli, see Section 2) as the detergents used to solubilize these proteins can disrupt protein interactions using AP-MS. Moreover, biotin labeling accumulates over time and the biotinylation mark remains on the prey, even if the protein has moved out of the vicinity of the PDB enzyme, leading to an increased chance to capture weak and transient interactions and generates a history of previous interactions (Roux, 2013; Varnaitė & MacNeill, 2016). As a disadvantage, the strong interaction of biotin with streptavidin can hamper elution of the biotinylated prey from the affinity support but the common practice of proteolytic digestion on the support to obtain peptides for MS analysis solves this problem.

Yeast two-hybrid (Y2H) is one of the most popular methods for detection of binary interactions (reviewed in Brückner et al., 2009; Snider et al., 2015) and enables high throughput experiments to test a large number of prey and bait combinations in yeast cells (Trigg et al., 2017). Y2H combines selectable reporter gene expression with cDNA library screens (Fields & Song, 1989). While Y2H is well suited to detect weak interactions, it also can result in high false-positive rates. Another problem with Y2H is that some interactions require more than two components or depend on modifications such as acylation, methylation, or phosphorylation (Duan & Walther, 2015). The successful detection of interactions relies on the correct expression and folding of the bait and the prey as well as their location in the same compartment. A genome-wide Y2H analysis exists for Plasmodium falciparum but first had to overcome problems due to the AT-rich genome of this parasite which hampered expression of full-length parasite genes in yeast and required expression of overlapping fragments (LaCount, 2012; LaCount et al., 2005). The capacity of Y2H to generate comprehensive interaction networks of entire organisms is an advantage over PDB methods but it is not suited to generate compartment proteomes and to obtain interaction histories of specific baits in the living organism of interest which are strengths of PDB.

The capacity of PDB to also provide compartment proteomes can be compared with organellar profiling techniques that enable the identification of subcellular localization of many proteins of either one organelle or all subcellular compartments of a cell at a time (reviewed in Borner, 2020; Lundberg & Borner, 2019). Proteins are
assigned to their subcellular localization after biochemical fractionation of cell lysates by machine-learning cluster analyses of protein abundance profiles, with the advantage that this is independent of genetic modifications of proteins, labeling properties of enzymes, organelle-targeting signals, or a particular bait to identify the proteins of a target compartment. Compared with PDB approaches that
allow for both, probing of fast dynamic processes (APEX, TurboID/miniTurbo) and the generation of a protein interactions history (BioID), organellar profiling provides a broad snapshot of compartment proteomes. PDB and organellar profiling can mutually benefit from each other, for example, PDB can yield proteomes of specific compartments that are not amenable to biochemical separation, and further, when several individual compartments in the cell are targeted by using multiple baits, PDB can provide whole cell maps similar to those from organellar profiling (Borner, 2020; Liu, Salokas, et al., 2018; Lundberg & Borner, 2019). Organellar profiling often faces problems if proteins are localized to multiple compartments which can lead to uncertain classifications or to achieve sufficient yields for less abundant organelles and protein complexes that require a sufficiently large quantity of cells (Lundberg & Borner, 2019).

Although this might represent a problem for some apicomplexan parasites, a comprehensive localization of organelle proteins by isotope tagging (LOPIT/hyperLOPIT; Mulvey et al., 2017; Sadowski et al., 2006) was recently provided for Toxoplasma gondii extracellular tachyzoites, revealing the protein distribution in an apicomplexan cell on an unprecedented scale (Baryllyuk et al., 2020; Koreny et al., 2021).

Some interaction analysis techniques are well suited to validate the outcome of large-scale analyses such as PDB experiments. To test for direct interactions of bait and prey, affinity purification methods (such as Co-IP or GST pull downs) are mostly used. Alternative suitable techniques to verify direct interactions are for example fluorescence PCA methods such as split-GFP that allows for direct visualization of binary protein interaction in the living cell (Kerppola, 2008; Remy & Michnick, 2004; Shekhawat & Ghosh, 2011; Zhang et al., 2016) and Förster resonance energy transfer (FRET), providing the possibility to detect protein interaction in living cells in real time (Ma et al., 2014). For monitoring the localization of the prey within the subcellular compartment of interest or the colocalization with the bait, simple fluorescent microscopy techniques are often sufficient.

2 | PDB IN APICOMPLEXA

The phylum of Apicomplexa comprises unicellular parasitic protozoa, many of which are pathogens that infect arthropods and vertebrates. They include the causative agent of human diseases like malaria (Plasmodium spp.), babesiosis (Babesia), cryptosporidiosis (Cryptosporidium spp.), and toxoplasmosis (Toxoplasma gondii), as well as diseases of livestock (Eimeria spp. and Theileria spp.).

In these unicellular parasites, the power of protein labeling with biotin is strengthened by the paucity of biotin ligases and their corresponding substrates. Plasmodium spp. parasites contain only two biotin ligases and Acetyl-CoA carboxylase (ACC) is the only predicted biotinylated protein. ACC is localized to the apicoplast but only biotinylated in liver-stage parasites, where it performs an essential function (Dellibovi-Ragheb et al., 2018; Müller & Kappes, 2007). T. gondii parasites contain only one biotin ligase which predominantly biotinylates two proteins, ACC and pyruvate carboxylase (Dellibovi-Ragheb et al., 2018; Jelenska et al., 2001). Inhibition of ACC in T. gondii parasites reduces parasite growth (Zuther et al., 1999). In contrast, while Cryptosporidium parvum parasites lack an apicoplast but express a cytosolic ACC and biotin ligase, Theileria annulata parasites lack both (Dellibovi-Ragheb et al., 2018). Overall, this suggests that some “background” biotinylation may occur that could limit detection efficiency in PDB experiments.

Several recent studies in Plasmodium spp. and T. gondii parasites as well as in Theileria annulata-infected host cells have successfully used PDB to identify novel proteins in subcellular compartments (Figure 2; Table 1). For Apicomplexa, BioID was first described in T. gondii tachyzoites to search for novel components of the inner membrane complex (IMC; Chen, Kim, et al., 2015). For this, the IMC-associated proteins ISP3 and AC2 were fused to biotin ligase as bait. This led to the identification of a hitherto unknown subset of proteins, named IMC suture components (ISCs) that are localized to the junctions between the alveolar sacs of the IMC (Chen, Kim, et al., 2015). In addition, using ISC4-BirA*, the ISC protein subset was extended, to further define IMC transverse sutures components (TSCs; Chen et al., 2017). Similar approaches, using the peripheral protein CDPK3 or several key components either associated with the apical complex or with the cyst wall of T. gondii parasites fused to BirA* or BioID2 led to proximity-based interactomes of proteins related to parasite egress and motility, the conoid, dense granules (DG), apical annuli, and the cyst wall (Gaji et al., 2015; Nadipuram et al., 2016; Long, Anthony, et al., 2017; Long, Brown, et al., 2017; Pan et al., 2019; Engelberg et al., 2020; Tu et al., 2020; Koreny et al., 2021). In addition, BirA*-based labeling of proteins localized to DG has also been applied to T. gondii bradyzoites using Mag1 as bait (Nadipuram et al., 2020). Expression of a fusion protein containing miniTurbo (Section 1.3) and a parasitophorous vacuolar membrane (PVM) targeting domain in the cytosol of human foreskin fibroblasts allowed the identification of unknown proteins at the PVM, facing the cytosolic host side of T. gondii parasites. This revealed a new set of proteins from the parasite as well as components of the host cell ESCORT machinery that might be involved in PVM establishment and maintenance (Cygan et al., 2021). A similar approach was taken in Theileria annulata-infected host cells. These parasites transform their host cells and link themselves to the microtubule cytoskeleton so that upon host cell division they distribute along with the mitotic spindle to the daughter cells (von Schubert et al., 2010). To achieve this feat, the parasite expresses proteins on the PVM that link to the host cell microtubules via the microtubule-binding proteins EB1 and CLASP1. To identify new parasite proteins involved in this link, the PVM targeting domain of CLASP1 was linked to BirA* (Huber et al., 2017, 2018). While the EB1-binding protein p104 was found to also directly interact with CLASP1, a newly identified protein TA03615 likely binds indirectly to CLASP1 (Huber et al., 2017).

Importantly, another newly identified protein called TaMISHIP was identified as part of a larger complex that regulates microtubules at the parasite PVM (Huber et al., 2018).
Several studies used PDB in the blood stages of the human and rodent parasites *P. falciparum* and *P. berghei* (Figure 2). PDB has been applied in different cellular compartments of *Plasmodium* asexual blood stages as well as in gametocytes and it has been used in vitro and in vivo in the mouse malaria model. This led to an expanded set of the known proteins resident in the apicoplast (Boucher et al., 2018) in the PV and at the PVM (Khosh-Nauke et al., 2018; Schneider et al., 2018), near the IMC (Wichers et al., 2021), inside egress vesicles of gametocytes (Kehrer et al., 2016), as well as in the GET machinery at the ER (Kumar et al., 2021).

While few modifications of PDB approaches have so far been used in apicomplexan parasites, DiQ-BioID, one of the conditional PDB systems (Section 1.3) was developed in malaria blood-stage parasites. Using DiQ-BioID, it was possible to identify proteins in a compartment with Kelch13, the protein mutated in artemisinin-resistant malaria parasites (Birnbaum et al., 2020). Of the identified Kelch13 compartment proteins, Ep15 was confirmed to be in a complex with Kelch13 by coimmunoprecipitation, highlighting the fact that BioID identifies both, proteins in the same compartment and interactors. The same study also used DiQ-BioID to detect proteins in the proximity of Ep15 and of clathrin. A further study used this technique to identify proteins localized to the rhoptries (Geiger et al., 2020).

In contrast to BirA*-based biotinylation, the faster peroxidase-based PDB (Section 1) is better suited for short-lived parasite stages and temporal snapshots of specific stages such as, for instance, to study aspects of cytokinesis, the egress out of the host cell, or the formation of microgametes in malaria parasites. However, particularly in malaria parasites, the potential of peroxidases and more active BirA variants have not yet been extensively exploited. Labeling with APEX2 has been used to determine the protein content of secretory vesicles in *P. berghei* ookinetes. To this end, the well-known micronemal protein SOAP was fused to APEX2, which led to the identification of known and novel putative micronemal proteins via MS analyses (Kehrer et al., 2020). In contrast to SOAP-APEX2, the fusion of SOAP to BirA* was not functional, possibly because of the long incubation time needed. Recently, *T. gondii* infected host cells transiently expressing APEX2 either in the cytoplasm or in the nucleus have been used to uncover effector proteins of the parasite secreted into the host cell cytoplasm or nucleus (Rosenberg & Sibley, 2021).

For direct comparison between the two methods, a complementary approach of BirA* and APEX or APEX2-based labeling has been used in *T. gondii* tachyzoites to identify novel proteins localized to mitochondria and DG, respectively (Pan et al., 2019; Seidi et al., 2018). PDB with BirA* and APEX/APEX2 was functional in both organelles but revealed interesting differences. In mitochondria, BirA*-based labeling efficiency was higher than with APEX but resulted in a higher number of contaminants (Seidi et al., 2018). In dense granules, APEX2-based labeling detected a higher number of proteins but appeared to be less specific than BirA* (Pan et al., 2019). Intriguingly, in both studies, <50% of all identified proteins were detected with both enzymes.

### 3 TECHNICAL CHALLENGES AND OUTLOOK FOR PDB IN APICOMPLEXANS

PDB has clearly helped to speed up research in Apicomplexan parasite biology. However, as with any technique, it also has some challenges and limitations (Table 2).

In contrast to some conventional methods used to identify protein–protein interactions, PDB nearly always involves genetic manipulation to tag a protein (see Section 1.4). Various tests should thus be done to ensure that protein-tagging does not influence protein function and localization. We usually first investigate if a GFP-tagged version of the protein of interest shows no phenotypic difference to the wild type. BirA* is slightly larger than GFP and although APEX and the optimized BirA*-derived ligases BioID2, BASU, and miniTurbo possess a similar size to GFP, we nevertheless recommend to also verify the life cycle progression capacity of the parasites used for proximity ligation. In addition, biotinylation of proteins is dependent on freely diffusing biotin throughout the parasite. This might result in reduced labeling efficiency in less accessible cell compartments (Kim & Roux, 2016). It is important to note that, depending on the target organelle, a significant proportion of enriched proteins can also be classified as contaminations from the secretory pathway because the bait is trafficked through the ER and Golgi network before arriving at the final destination. Natural biotinylation in mammalian cells generally occurs in the cytoplasm or mitochondria with a neutral or slightly basic pH. In contrast, the food vacuole of the parasite is strongly acidic (Homewood & Neame, 1974) and most likely an unsuitable environment for biotinylation. Compartments of the secretory pathway, such as ER, Golgi, and secretory vesicles, are only mildly acidic and their milieu does not seem to impair biotinylation (Wu et al., 2001; Kehrer et al., 2016, 2020; Geiger et al., 2020; Kumar et al., 2021). Yet, in mammalian cells, biotinylation of the ER lumen was more efficient using TurboID compared with classic BirA* (May et al., 2020). Furthermore, in cell culture-based experiments in vitro, there can be a significant amount of constant background biotinylation in cell expressing the bait as a result of biotin being present in the cell culture medium. Since biotin is redundant for the development of *Plasmodium* blood-stage parasites, this background can be reduced by using a biotin-free medium (Dellibovi-Ragheb et al., 2018; May et al., 2020). In short-lived parasite stages, such as *Plasmodium* ookinetes, BirA*-based PDB is problematic since the labeling takes more time than the development of the motile cell. Thus, biotinylation within ookinetes seems more efficient using the faster-acting peroxidase APEX2 (Kehrer et al., 2020). Optimized BirA* versions, such as TurboID or miniTurbo, with labeling times around 10 min (see Section 1.3; Branon et al., 2018) have not yet been used in *Plasmodium* parasites and might be alternatives to APEX2. Importantly, in *T. gondii*, miniTurbo has been shown to be functional (Cygan et al., 2021).

While ookinetes can successfully be produced in vitro outside the mosquito vector, the development of infectious sporozoites exclusively occurs within the insect. This limits the use of biotinylation, as multicellular organisms, including mosquitoes, contain many biotin ligases to facilitate natural biotin labeling. This, at least in our
hands, leads to an increased background, masking the fewer parasite proteins that are labeled by the PDB enzyme. Improved purification protocols or methods to grow sporozoites in vitro could improve labeling. If labeling is done in vivo, for example by feeding mosquitoes via sugar or salt pads, it is not clear how much of the administered biotin reaches the parasite-containing mosquito compartments such as the salivary glands. Here, the property of BioID2 to require less external biotin could be useful. Another key factor influencing BirA*-based labeling is the temperature (see Section 1.2).

While enzyme activity of the ligase is optimal at 37°C or above (Kim et al., 2016), ookinetes and sporozoites develop at around 20°C. This problem might be solved using TurboID/miniTurbo or APEX2, which have a broader temperature spectrum at which labeling can occur (May et al., 2020).

In Plasmodium blood-stage parasites, BirA*-based proximity labeling was successfully used in multiple approaches and laboratories. Yet, similar to above, the long labeling times might result in less-specific labeling. Again, using TurboID or miniTurbo might...
improve temporal resolution. Using APEX2 in blood stages might need some adjustment to the original protocol, due to the dissociation of hydrogen peroxide into hydrogen and water by the enzyme catalase in the blood. Extensive washing and sequential lysis of the red blood cell membrane before adding hydrogen peroxide might allow effective use of APEX-based biotinylation, although this would come at the cost of one of the key advantages of PDB, the capacity to carry it out in growing parasites.

4 | CONCLUDING REMARKS

Proximity labeling methods are functional in apicomplexan parasites and serve as powerful tools to obtain novel insights into defined cell compartments or special protein complexes. Essential thoughts for an effective experimental design should include the choice of the proximity labeling method as well as appropriate controls to determine labeling specificity. If possible, a combination of BirA*- and APEX-based biotinylation might be beneficial and could result in complementary data sets. Finally, like other interactome discovery methods, it is essential to appropriately validate the hits of PDB experiments. As always in science when using a nascent and quickly evolving technology, a new user will benefit from consulting with people who have used the technique and know the pitfalls before launching expensive experiments.

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Glossary

Apicoplast, Nonphotosynthetic chloroplast-like organelle.; Biotin, An essential vitamin for all organisms and involved in a wide range of metabolic processes.; Biotin ligases, Enzymes that catalyze the covalent attachment of biotin to usually specific biotin-dependent carboxylases.; Bradyzoite, Slow-growing parasite form in tissue cysts in the dormant stage of certain apicomplexans including T. gondii.; Conoid, Ring-like structure at the apical tip of motile parasite stages from which subpellicular microtubules spread out.; Crosslinking, Method to study interactions between proteins that involve covalent attachment of one protein to other proteins or solid support via a small crosslinking reagent.; Dense granules, Subset of secretory vesicles containing proteins destined for the PV, PVM, and host cell.; Egress vesicles, Secretory vesicles in gametocytes essential for egress from red blood cells.; GET machinery, Proteins of a pathway mediating the insertion of tail-anchored membrane proteins into the ER.; Inner Membrane complex (IMC), Golgi-derived flattened compartment, underlying the plasma membrane in around 25 nm distance.; Interactome, Network of all protein interactions in a cell used in conjunction with a particular bait and refers to all possible interactions of that bait.; Kelch13, P. falciparum protein causing Artemisinin resistance when mutated.; Mass spectrometry (MS), Commonly used analytic method for the identification of molecules (purified biotinylated proteins in PDB) by measuring the mass-to-charge ratio of ions.; Micronemes, Specialized vesicles concentrated at the apical part of invasive parasite stages. Its secreted proteins are needed for parasite adhesion and host-cell invasion/trans- versal and egress.; Ookinetes, Motile zoogotes of Plasmodium formed in the mosquito midgut lumen. Once formed, they traverse the midgut epithelium where they transform into sporozoite-producing oocysts.; Parasitophorous vacuole (PV), Niche formed during and after host-cell invasion wherein the parasite develops during intracellular growth.; Peroxidases, Large group of enzymes that catalyze the oxidation of a substrate by hydrogen peroxide and thereby the biotinylation of proteins in the context of PDB.; Parasitophorous vacuolar membrane (PVM), membrane of the PV derived from the host cell membrane and rhoptry lipids; the PVM forms the interface to the host cell.; Rhoptries, Pear-shaped or elongated secretory organelles at the apical part of invasive parasite stages but not ookinetes. Proteins secreted from the rhoptries are involved in host-cell invasion and support the formation of the PV.; Streptavidin, Protein that binds to biotin with very high affinity.; Tachyzoite, Rapidly growing parasite stage, for example, during the acute phase of Toxoplasmosis.

CONFLICT OF INTEREST

The authors declare to have no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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