Proximity labeling: spatially resolved proteomic mapping for neurobiology

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Understanding signaling pathways in neuroscience requires high-resolution maps of the underlying protein networks. Proximity-dependent biotinylation with engineered enzymes, in combination with mass spectrometry-based quantitative proteomics, has emerged as a powerful method to dissect molecular interactions and the localizations of endogenous proteins. Recent applications to neuroscience have provided insights into the composition of sub-synaptic structures, including the synaptic cleft and inhibitory post-synaptic density. Here we compare the different enzymes and small-molecule probes for proximity labeling in the context of cultured neurons and tissue, review existing studies, and provide technical suggestions for the *in vivo* application of proximity labeling.

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Introduction

To study how spatially compartmentalized protein networks assemble into functionally integrated macromolecular complexes, we and others have developed a class of methods termed 'proximity labeling' (PL). PL uses engineered enzymes to selectively and covalently tag neighboring proteins with biotin in living cells [1••,2••]. The biotinylated proteins can then be isolated after cell lysis and characterized by mass spectrometry (Figure 1a). PL has been applied to map novel components of cellular organelles [3•,4–10] and to identify new protein-protein interaction partners with high spatial specificity [11–13]. These studies have demonstrated that PL is a powerful approach to dissect the interactions and localization patterns of molecules with nanometer spatial resolution [14,15].

Because of the complexity and heterogeneity of neurons in both spatial and temporal dimensions, PL also has the potential to benefit neuroscience research. For example, there is tremendous interest in mapping the molecular composition of synaptic subdomains (e.g. active zone, post-synaptic density, cleft, synaptic vesicles) and understanding how these change during plasticity and in disease [16]. At the level of the organism, PL could potentially be used to map the proteomic signatures of different brain regions, cell types, and synapse types [17]. For specific proteins of interest, such as ion channels and receptors, PL could yield novel interaction partners that play important functional roles in regulation or signaling [18].

Yet there have been very few examples of PL applied to neuroscience to date. In this review, we summarize the few existing studies, analyze the technical challenges to the broader application of PL to neuroscience, and envision future applications.

Enzymes used for proximity labeling

Proximity labeling methods can be divided into two categories based on the enzyme used to carry out the catalysis: peroxidase-based PL and biotin ligase-based PL. Peroxidase-based PL relies on expression of an engineered ascorbate peroxidase (APEX or APEX2) [19,20•] or horseradish peroxidase (HRP) in the cells or tissues of interest. Alternatively, HRP can be targeted to specific cell surface antigens via conjugation to an antibody [21,22]. To initiate labeling, H_2O_2 is added for 1 minute to cells/tissues preloaded with the substrate biotin-phenol (BP) [1••] (or its variants, such as BxxP [23••], alkyne-phenol [1••], and desthiobiotin phenol [24]). The peroxidase oxidizes BP into a phenoxyl radical that reacts with nearby proteins at electron-rich side chains (Figure 1b), a mechanism similar to the commonly used tyramide signal amplification (TSA) kit. As the phenoxyl radical has a half-life of less than 1 ms [25], the labeling intensity dramatically falls off within nanometers from the peroxidase active site, generating a biotinylation contour map that is then read out by quantitative proteomics to give a ranked protein list based on proximity to the enzyme. For example, using APEX targeted to the outer mitochondrial membrane (OMM), proteins residing on the OMM could be readily distinguished from immediately adjacent cytosolic proteins [20•].

The rapid kinetics of the peroxidase reaction (<1 minute labeling time) can be harnessed to interrogate dynamically evolving protein interaction networks. Two recent studies used APEX fusions to G-protein coupled

Figure 1

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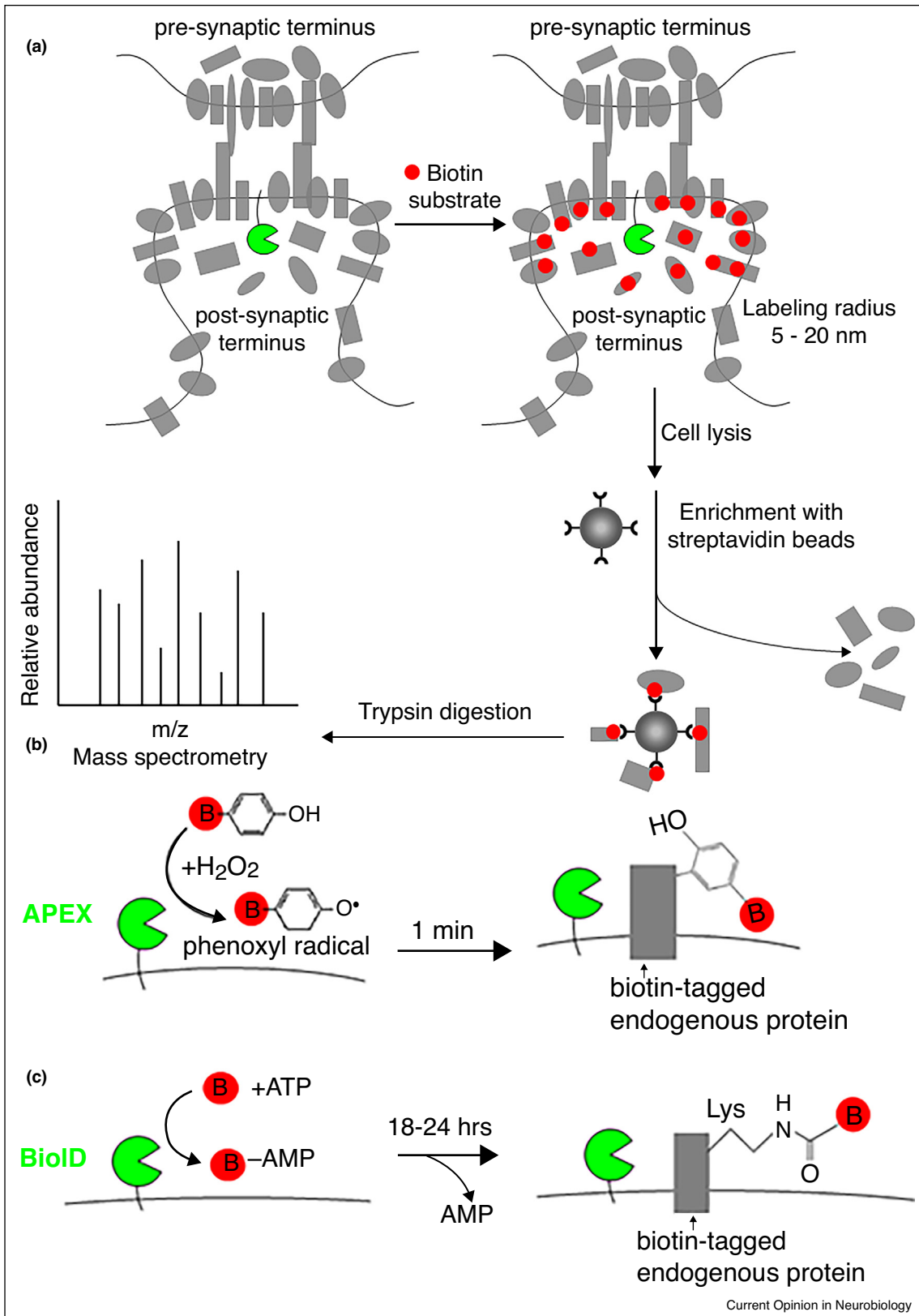
receptors (GPCRs) to map protein interaction partners at various timepoints after ligand stimulation [26,27•]. Because HRP/APEX can also survive cell fixation and oxidatively polymerize diaminobenzidine, these enzymes can also be used to generate contrast in fixed cells for electron microscopy (EM), enabling users to examine peroxidase fusion constructs for proper subcellular localization before initiation of a proteomic study [19].

The major distinction between APEX and HRP is that HRP is only active in the secretory pathway and extracellular environment due to its need for disulfide bond formation [19]. However, HRP is more active than APEX2, which makes it a superior choice in the compartments in which it is active [23••].

The enzyme BioID, which is an R118G mutant of *Escherichia coli* biotin ligase (BirA), is used in biotin ligase-based PL [2••]. BirA requires both biotin and ATP as substrates to generate reactive biotinoyl-5′-AMP (bioAMP), which it then transfers onto specific lysine residues on bacterial carboxylase proteins. The R118G mutation in BioID reduces BirA's affinity for bioAMP, resulting in release of the reactive intermediate to promiscuously tag lysine residues on proximal proteins [28] (Figure 1c). Although the half-life of bioAMP is many minutes in water [29], BioID has been shown to have a labeling radius of ∼10 nm from mapping of the nuclear pore complex [11], indicating that the half-life is likely to be much less inside cells, perhaps due to the high density of intracellular nucleophiles. A major limitation of BioID compared to peroxidase-based PL is that, due to its slow kinetics, BioID requires 18–24 hours of reaction time to obtain sufficient labeled material for proteomics. The long labeling timescale makes BioID non-optimal for the study of dynamic processes. In addition, BioID fusion constructs may mistarget more than APEX fusion constructs, due to its larger size (35 kDa compared to 28 kDa for APEX); mistargeting has previously been observed for BirA fusions [30]. Recently, a smaller version (27 kDa) of BioID, BioID2, was reported [31•].

Applications of proximity labeling to neuroscience

Recent studies have demonstrated the value of PL in elucidating the molecular components of synaptic clefts and the inhibitory postsynaptic density (iPSD). Despite long-standing interest in defining the molecular components that mediate information flow between communicating neurons, biochemical purification of many synaptic subregions remains intractable.

Loh et al. used HRP peroxidase in living neurons to map the proteomes of both the excitatory and inhibitory synaptic clefts, which are impossible to purify biochemically, and identified the glycosylphosphatidylinositol anchor protein Mdga2 as a potential specificity factor influencing Neuroligin-2's recruitment of presynaptic neurotransmitters at inhibitory synapses [23••]. To adapt previous peroxidase labeling protocols used for cancer cells to cultured neurons, a new lysis and enrichment protocol was developed to disassemble the detergent-insoluble and tightly crosslinked post-synaptic density (PSD), by adding a high percentage SDS lysis step with 10 minutes of boiling. This removed cytosolic contaminants co-purifying with biotinylated cleft-exposed proteins. Another methodological advance in this study was the application of an intersectional labeling strategy, using two independent peroxidase fusion constructs (HRP-Lrrtm1 and HRP-Lrrtm2 for excitatory cleft, and HRP-Nlgn2A and HRP-Slitrk3 for inhibitory cleft) targeting the same cellular locale, in order to improve the specificity of protein identifications. Finally, because non-dividing cells such as neurons are not amenable to SILAC (stable isotope labeling with amino acids in cell culture) labeling, which requires cell division and/or high protein turnover for metabolic incorporation of labels, protein quantitation was instead achieved through iTRAQ (isobaric tags for relative and absolute quantitation) chemical labeling. TMT (tandem mass tags) chemical labeling can also be used for quantitation in non-dividing cells. A similar workflow was applied to map the alpha-synuclein interactome in living neurons using APEX [13].

The iPSD is the only subcellular structure that has been investigated by PL in the brain of a living animal [32••]. The application of BioID in the living mouse brain represents a major milestone for PL in neuroscience, as probe delivery and labeling procedures significantly differ from cell culture. To express BioID-fused proteins *in vivo*, adeno-associated viruses encoding biotin ligase fusion proteins were injected into the cortex and hippocampus of postnatal mice. The exact virus injection times, biotin dosage and labeling times were carefully optimized to achieve maximum number of synapses

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(Figure 1 Legend) Workflow and mechanism of proximity labeling. **(a)** An engineered enzyme (green) is genetically targeted to the subcellular region of interest (e.g. the iPSD) and covalently tags proximal endogenous proteins with a biotin handle. The gray shapes are endogenous proteins residing inside and outside the region of interest. Following cell lysis, biotinylated proteins are enriched with streptavidin beads, digested to peptides on-bead, then analyzed by liquid chromatography and tandem MS. **(b)** For peroxidase-based labeling using APEX, H_2O_2 is added for 1 minute to cells preloaded with biotin-phenol (BP; red B = biotin) to initiate labeling. APEX oxidizes BP into a phenoxyl radical, which covalently tags proximal endogenous proteins at electron-rich side chains such as tyrosine. **(c)** For biotin ligase-based labeling using BioID, exogenously supplied free biotin is utilized together with endogenous ATP for 18–24 hours of labeling. BioID converts biotin to reactive bioAMP, which is released from the enzyme's active site to react with lysine residues on proximal proteins. Both bioAMP and the biotin phenoxyl radical do not cross cellular membranes.

labeled. As biotin delivery into mouse brain is more challenging than in cell culture, the authors opted for intraperitoneal administration of biotin, which can penetrate various tissue types including the blood-brain barrier [33]. The mice were then labeled for 7 days before harvesting biotinylated material. Hence, temporal specificity for BioID *in vivo* is still lacking. Nevertheless, the study identified and validated many previously unknown components of the iPSD, including InSyn1 and InSyn2. Knockout of InSyn1 led to decreased postsynaptic inhibitory sites, reduced the frequency of miniature inhibitory currents, and increased excitability in the hippocampus, highlighting the power of PL to reveal new neurobiology.

In vivo application of PL requires probe delivery into tissue

Since implementation of PL requires delivery of both a genetic component (DNA encoding the enzyme) and a chemical component (the small-molecule substrates) to cells, an important consideration when performing PL *in vivo* is how to deliver the substrate molecules to the relevant organs or tissue. For the iPSD study described above [32••], biotin was delivered to the brain via intraperitoneal injection. Mammals do not synthesize their own biotin, but it is an essential vitamin used for fatty acid biosynthesis. Import of biotin at low concentrations ($<5 \mu M$) into cells is mediated primarily by the Na^+-dependent multivitamin transporter (SMVT1), which is ubiquitously expressed in various tissues including the intestine, liver, brain, heart, lung and kidney. Passive diffusion across membranes occurs when biotin concentration exceeds $25 \mu M$ [34]. Taking advantage of these entry routes, biotin for BioID can be supplied through animal food in principle. However, this method of delivery may lead to substantial variation in labeling among individual animals as the food intake behavior may vary. The iPSD study used daily intraperitoneal injection to supply exogenous biotin, providing a simple and efficient way to control biotin dosage. Importantly, biotin is not known to be toxic even at high doses; when administered to people without biotin metabolism disorder at up to 5 mg/day for two years, adverse effects were not observed [35].

Apart from this study, one other *in vivo* BioID example has been reported. Here, the authors investigated c-MYC interaction partners in a tumor xenograft model, where tumor cells expressing BioID were injected into mice [36]. Again, exogenous biotin was supplied via intraperitoneal injection.

In some cases, users may opt for peroxidase-based PL *in vivo*, to better control the time window of labeling. *In vivo* substrate delivery for APEX-based PL is more challenging than for BioID, but a few recent studies have succeeded [37–39]. In insects and worms, the tightly sealed and hydrophobic cuticle acts as a protective outer layer

and is highly impermeable to small molecules in the environment. Two recent studies applying APEX proteomics to the intestine of living *C. elegans* [38,39] used RNAi knockdown of a glycosyltransferase gene, *bus-8*, to compromise cuticle integrity and enable the delivery of BP and H_2O_2 to the worm interior. However, disrupting a gene with known developmental functions is a concern for both the proteomic experiment and data interpretation. An alternative strategy is to dissect the tissue of interest from the intact animal body, as employed in an APEX study in *Drosophila* muscle mitochondria [37]. Dissection and labeling in proper tissue culture media maintains the tissue in a physiological environment for the short duration of the H_2O_2 reaction, providing a potentially generalizable strategy for *in vivo* labeling with high temporal resolution. The downside of this approach is that the dissection process may be labor-intensive and difficult for certain tissue types.

Although H_2O_2 is used at a low concentration in the APEX PL reaction (1 mM) and only for a short period of time (<1 minute), H_2O_2 delivery could lead to undesired cytotoxicity arising from oxidative stress signaling. 1 mM of H_2O_2 has been shown to induce apoptosis in 24 hours through activation of p53-related pro-oxidant gene expression such as BAX, PIG3 and PUMA [40]. Sub-millimolar concentrations of H_2O_2 for just 15 minutes can cause significant aldehydic DNA lesions in HeLa cells [41]. Therefore, restricting H_2O_2 labeling to low concentrations and short time windows is crucial for minimizing side effects. For tissues with minimal thickness and size, such as whole worms or dissected *Drosophila* muscles, H_2O_2 can quickly penetrate and react throughout the entire sample, just as it does in cell culture [37–39]. In contrast, immersing the whole mouse brain in H_2O_2 solution is less likely to be effective, because by the time H_2O_2 penetrates the center of the tissue mass, too much damage may have been caused to the cortex by oxidation. Acute slice preparations in artificial cerebrospinal fluid, such as for slice electrophysiology, could be considered for vertebrate brain samples to avoid long incubation times in H_2O_2.

The challenge of high background *in vivo*

Another technical challenge to applying PL *in vivo* is that tissue contains more substantial sources of background than cultured cells. The background can come from two sources: non-specific binding of material to streptavidin-coated beads, and endogenous biotinylated proteins. The former problem arises when the experimentalist is infecting only a small fraction of the total cells in a tissue region or organism, or when the proteome of interest is very small and localized. Then the ratio of biotinylated (desired) material to non-biotinylated (undesired) material is very small, and it is difficult to implement a successful enrichment. To overcome this, crude fractionation of the sample before streptavidin enrichment may

help to increase the ratio of desired to undesired material. For example, if conducting a PL experiment targeting a sub-synaptic region, a crude synaptosome preparation [42] before streptavidin enrichment could help remove background proteins from unrelated regions. Dissecting out organs of interest before homogenization and streptavidin enrichment could also be beneficial.

The second source of background, endogenous biotinylated proteins, also becomes a serious problem when working with small fractions of transfected cells, or small and localized proteomes. In the *Drosophila* brain, for example, endogenous biotinylated proteins are highly abundant, especially in glia cells [43]. These endogenous biotinylated species can vastly exceed the BioID- or APEX-biotinylated proteomes in mass, and compete for binding to streptavidin beads. Again, fractionation or dissection may help in these cases by removing background from unrelated regions. It would also be valuable in the future to develop non-biotin-based strategies for enrichment of tagged proteomes. In Rhee et al. [1••], for instance, it was shown that APEX can label proteins with alkyne-phenol instead of biotin-phenol. Alkyne can be 'clicked' to various azide reagents [44], potentially enabling enrichment without the use of streptavidin beads.

Tissue presents other sources of background as well. For peroxidase-based PL, background can arise from the activity of endogenous peroxidases. For example, in the *C. elegans* intestine, it was found that endogenous peroxidase activity is higher than that typically observed in cultured cells [39]. Therefore, designing experiments with both unlabeled and untransfected controls is essential for minimizing interference from non-specific binders, endogenous biotinylated proteins, and endogenous peroxidase activity.

Another strategy to overcome background in tissue is to increase the desired signal. For example, one could extend the reaction time to label more material, as in the iPSD *in vivo* study [32••] and in a *C. elegans* study that used 2 minutes instead of the typical 1 minute for APEX tagging [39]. For peroxidase-based PL in particular, sometimes low signal results from high expression of catalases, which quench H_2O_2, or from low availability of the peroxidase co-factor heme, which can be improved by heme supplementation [45].

Outlook for the application of proximity labeling in neurobiology
In the last decade, proteomics in combination with biochemical approaches (e.g. fractionation, immunoprecipitation, and chemical crosslinking) has been instrumental in characterizing the molecular components of many neuronal structures, including entire synaptic terminals [42], synaptic vesicles [46], the PSD [47] and the active

zone [48]. Advanced imaging approaches, such as array tomography [49], have offered neuroscientists high-resolution maps of molecular architecture in the brain. However, array tomography is limited to only ∼20 targets at once, as well as antibody availability, while purification-based proteomics suffers from high false-positive rates due to contamination. For instance, intracellular contaminants such as mitochondrial and nuclear proteins account for 20–40% of identifications in synaptosome preparations [42]. Therefore, PL-based proteomic maps with high spatial and temporal resolution would greatly complement these existing techniques.

Besides the subcellular regions already mapped with PL-based proteomics, neurons harbor many other functionally specialized subcellular structures, such as the growth cone for axon guidance [50] and the axon initial segment for signal integration [51], as well as highly distinct structures executing unique functions in certain cell types. For example, electrophysiological and structural evidence has indicated that stereocilia tips of hair cells, which are the primary mechanical sensors for hearing in mammalian ears, house the elusive mechano-to-electric transduction machinery with unknown molecular identity [52]. PL-based proteomic profiling provides an unprecedented opportunity to systematically map the protein composition of these functionally specialized regions. Notably, growing evidence suggests that glia are more than supportive cells, but are extensively involved in regulating the development and function of the nervous system as well [53]. Proteomic mapping of the interface between glia and neurons would further our understanding of the molecular basis of glial-neuronal interaction, such as the signal controlling myelination. Furthermore, glial pathogenesis has been implicated in many neurological disorders such as Alzheimer's [54,55]. *In vivo* PL-based proteomics of different cellular compartments (e.g. endosomes, lysosomes, mitochondria, cell surface) in different types of glia (astrocytes, microglia, oligodendrocytes) under healthy and pathological conditions would provide a means to gain molecular insights into the role of glial cells in diseases.

Proteomic mapping of dynamic interactomes offers another exciting avenue to advance our understanding of key molecules in neurobiology. Although a huge collection of molecules essential for neural development [56–58] and signal transmission [59] has been identified through genetic analysis, most are still 'orphan' genes, for which we have limited knowledge of interaction partners and function. Applying PL-based proteomics to these molecules could capture both stable and transient partners and yield a comprehensive interaction network. Moreover, the high temporal resolution of peroxidase-based PL would allow us to profile interaction dynamics under different conditions, as demonstrated in the GPCR pathways [26,27•]. As many of these molecules are linked

with human diseases [60], systematically profiling their interacting partners under disease conditions would not only shed light on the pathological mechanisms but also potentially identify novel disease-associated genes.

Conflict of interest statement

A.Y.T. is an author of a patent application on the peroxidase technology.

Acknowledgements

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•• of outstanding interest

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