Use of Proximity Labeling in Cardiovascular Research

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HIGHLIGHTS

- Protein interaction networks are of critical importance in regulating cardiac physiology, including adrenergic signaling.
- The proximity labeling approach is based on the enzymatic-dependent conjugation of biotin to endogenous proteins in intact cells, enabling their selective enrichment and identification.
- Proximity labeling and mass spectrometry have been used to identify novel protein-protein interactions in cardiac and vascular cells.
- The use of proximity proteomics is highlighted in the discovery of the mechanism underlying adrenergic regulation of Ca^{2+} influx in the heart.

SUMMARY

Protein-protein interactions are of paramount importance in regulating normal cardiac physiology. Methodologies to elucidate these interactions in vivo have been limited. Recently, proximity-dependent biotinylation, with the use of BioID, TurboID, and ascorbate peroxidase, has been developed to uncover cellular neighborhoods and novel protein-protein interactions. These cutting-edge techniques have enabled the identification of subcellular localizations of specific proteins and the neighbors or interacting proteins within these subcellular regions. In contrast to classic methods such as affinity purification and subcellular fractionation, these techniques add covalently bound tags in living cells, such that spatial relationships and interaction networks are not disrupted. Recently, these methodologies have been used to identify novel protein-protein interactions relevant to the cardiovascular system. In this review, we discuss the development and current use of proximity biotin-labeling for cardiovascular research.

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Proximity proteomic methodologies have been developed to enzymatically label nearby proteins while still maintaining information about the proteins’ location and interactions with other proteins within a cell (1-6). With the rapid advancement of proximity labeling techniques comes the ability to collect substantial data and answer longstanding scientific questions that would otherwise have been impossible. In this review, we discuss using these techniques for cardiovascular research, highlighting the use of proximity labeling and quantitative mass spectrometry to identify the...
mechanisms underlying adrenergic regulation of $Ca^{2+}$ current in the heart (7).

**DEVELOPMENT OF PROXIMITY-DEPENDENT BIOTINYLA TION METHODOLOGY**

With the advent of transcriptomics and the development of extensive lists of candidate genes, identifying critical protein interactors has remained a serious bottleneck in advancing our understanding of physiology and pathophysiology. To elucidate the components of macromolecular complexes, biochemical fractionation followed by mass spectrometry can be performed (8-10). Another approach uses affinity purification of a protein, typically by antibody, followed by mass spectrometry (11). These methodologies, however, have important limitations (12,13). Some proteins, especially membrane-bound proteins, are relatively inaccessible and difficult to solubilize. Coimmuno-precipitations require high-quality antibodies that are often not available. Important interactions may have fast-off kinetics and therefore will not persist after solubilization and during affinity purification (14). Also true of yeast-hybrid approaches, the biological process of interest may only operate in the proper milieu, as part of a larger more extensive protein interaction network that only exists in terminally differentiated mammalian cells. In addition, tissue-specific post-translational modifications can reduce the effectiveness of these methods.

Cognizant of these limitations, Roux et al. (15) adapted an *Escherichia coli* biotin ligase, BirA, to perform in situ labeling in eukaryotic cells. This system has several advantages. Posttranslational modification with biotin, that is, vitamin B$_7$, was chosen in part for its low toxicity and frequent use, alongside its ultra-high-affinity binding partner streptavidin, in molecular biology and biochemistry. There is also a relative paucity of biotin in eukaryotic cells: generally, there is only a single biotin ligase per species and these are known for their exquisite specificity, targeting only a single lysine residue in up to 5 different protein targets depending on the species (16). Roux et al. (15) fused different variants of BirA to lamin A, a nuclear envelope intermediate filament protein, mutation of which can cause a number of diseases, including dilated cardiomyopathy (17). In HeLa and human embryonic kidney (HEK) 293 cells, BirA R118G, also known as BirA* and named BioID for “biotin identification,” was especially promiscuous. This variant was capable of a marked increase in nonspecific but proximity-based biotin ligation with the addition of excess biotin to the culture media (Figure 1A) (15). Combined with streptavidin-based affinity purification of biotinylated proteins and liquid chromatography/mass spectrometry, numerous known and novel interactors of lamin A were identified (Central Illustration). Depending on the experimental conditions and labeling time, the proximity-labeling radius may be as low as a few nanometers (4). For these reasons, BioID has been used in diverse model systems, and within 4 years of its introduction it was coupled with injection of adeno-associated virus to characterize a tissue-specific subcellular proteome in mice in vivo: post-synapse cortical inhibitory neurons in newborn mice (18).

The next generation of biotin ligases overcame at least some of the limitations of BioID, especially its slower kinetics, which requires longer labeling times. Branon et al. (4) used error-prone polymerase chain reaction and 5 generations of yeast display-based directed evolution to produce a more promiscuous and faster BirA, TurboID, capable of markedly greater biotin labeling in heterologous expression and in live *Caenorhabditis elegans* and *Drosophila melanogaster*. An alternate fifth-generation BirA contained an N-terminal truncation with slightly less activity than the full-length form but also decreased labeling in the absence of biotin supplementation (referred to as miniTurbo). This has important implications in the development of transgenic animals. For example, the fraction of fruit flies expressing miniTurbo surviving to adulthood did not differ from control; however, fewer than one-half of TurboID-expressing flies survived to adulthood, an effect that was completely eliminated with dietary supplementation of biotin.

One important limitation of BioID proximity labeling is the time required to detectably biotinylate neighboring proteins—hours to days of biotin supplementation. This limitation has been somewhat reduced with the advent of TurboID, although this engineered biotin ligase still requires a minimum of several minutes to biotinylate protein neighbors (4).

The introduction of soybean ascorbate peroxidase (APEX) and its engineered derivatives represented a major advance in the field with the generation of significant enzyme-catalyzed proximity labeling within seconds, allowing the capture of dynamic signaling events at high resolution with subcellular localization (1,2). APEX2 was engineered with yeast display-based evolution to improve its catalytic efficiency, increasing sensitivity in cells with lower transgene expression. In the presence of biotin-
tyramide (biotin-phenol) and hydrogen peroxide, these peroxidases form biotin-phenoxyl radicals that form covalent linkages to electron-rich residues (primarily tyrosines; Figure 1B) (1). The short half-life of these radicals (<1 ms) and their rate of diffusion ensures that labeling occurs only within ~20 nm of APEX (19). Addition of an antioxidant cocktail quenches the reaction. The membrane impermeability of these radicals ensures that proximity labeling is limited to membrane-contained or membrane-excluded spaces.

There are several limitations, namely that the labeling by APEX2 requires hydrogen peroxide, which can induce brief oxidative stress, and that the high labeling activity of APEX2 may preclude the precise spatial resolution required to identify the local interaction network of a target protein (20). For example, clouds of APEX2-generated biotin-phenoxyl
FIGURE 1 Proximity Proteomics Labeling Methods Used in Cardiovascular Studies

(A) Biotin labeling of sterically available basic residues by the Escherichia coli biotin ligase BirA, or engineered derivatives (BirA* [BioID], BioID2, TurboID, and miniTurbo). BirA* is conjugated to the protein of interest. With biotin supplementation, biotin is covalently linked to both interacting and neighboring proteins.

(B) Soybean ascorbate peroxide (APEX) or its derivative (APEX2) is conjugated to the protein of interest, which catalyzes the generation of biotin phenoxyl radicals that form covalent linkages with sterically available electron-rich residues. Proteins within the "cloud" of biotin phenoxyl radicals are labeled with biotin, whereas those outside of the radius are not labeled.

(C) Complementation of N- and C-terminal APEX2 fragments, "AP" and "EX," respectively, induced by rapamycin-mediated interaction between FK506 binding protein (FKBP) and its binding partner, the FKBP-rapamycin binding (FRB) domain of the kinase mammalian target of rapamycin. Biotin labeling of neighboring proteins is enabled in cellular locations where both protein A and protein B interact. Figure created with BioRender.com.
radicals may surround APEX2 fusion proteins in various subcellular compartments, not limited to the ultimate trafficking destination of the protein of interest, tagging bystander proteins along the way (e.g., ribosome and rough endoplasmic reticulum [ER], smooth ER, Golgi network, and so on). Proteins in these compartments may be important for folding, trafficking, and/or degradation, but deconvolving...
these neighborhoods is challenging. To address these limitations, quantitative proteomics were combined with either spatial references (20) or isobaric tandem mass tags (21) to enable tracking the activation, internalization, and subcellular protein complexes of G-protein–coupled receptors when expressed in HEK cells.

**USE OF APEX-BASED PROXIMITY PROTEOMICS IN THE HEART**

Ca\(^{2+}\) channels, which are the major pathway for entry of Ca\(^{2+}\) ions into excitable cells, transduce membrane depolarization into multiple cellular functions (22). In the heart, the influx of Ca\(^{2+}\) is essential for activation of excitation-contraction coupling and modulating pacemaker activity in sinoatrial nodal cells. In adult ventricular cardiomyocytes, the predominant type of Ca\(^{2+}\) channel, Ca\(_{\text{v}}\)1.2, localizes to transverse tubules in close proximity to ryanodine receptors at dyadic junctions (23). Despite considerable research, the molecular mechanisms responsible for adrenergic stimulation of Ca\(_{\text{v}}\)1.2 have remained stubbornly obscure for decades (24–30). For many years, the mechanism was thought to involve protein kinase A (PKA) phosphorylation of residues on Ca\(_{\text{v}}\)1.2 \(\alpha\)1C- and/or \(\beta\)2B-subunits (31–37). More recent studies in mice demonstrated, however, that adrenergic stimulation of Ca\(_{\text{v}}\)1.2 did not involve direct phosphorylation of these residues on \(\alpha\)1C or \(\beta\)2 subunits (Figure 2A) (7,38–44).

There are many hundreds of proteins localized to the dyadic space, many of which could transiently interact with the Ca\(^{2+}\) channel and serve as the functional PKA target (Figure 2A). Based on the observations that \(\beta\)-adrenergic regulation of Ca\(_{\text{v}}\)1.2 cannot be reconstituted when the core subunits are heterologously coexpressed (45), that PKA phosphorylation of the \(\alpha\)1C- and/or \(\beta\)2B subunit is not sufficient (43), and that Ca\(_{\text{v}}\)1.2 is the epicenter of a large macromolecular complex, we aimed to identify proteomic neighbors of Ca\(_{\text{v}}\)1.2 in the heart that may be the true PKA target. The specialized ultrastructure of the cardiac dyad is spanned and maintained by junctophilin-2 (Jph2), which brings the sarcoplasmic reticulum and its Ca\(^{2+}\) release channels within 12–18 nm of the transverse tubules and its membrane-bound Ca\(_{\text{v}}\)1.2, permitting excitation-contraction coupling with every single heart beat (46–49). It is therefore a membrane-enclosed space well suited to proximity-based proteomic characterization with the use of proximity labeling.

Transgenic mice with doxycycline-inducible cardiomyocyte-specific expression of dihydropyridine-resistant \(\alpha\)1C or \(\beta\)2B proteins with APEX2 and a V5 epitope conjugated to the N-terminals were generated (Figure 2B). This inducible transgenic methodology (50,51) was perfectly suited for these experiments as we could both temporally and spatially limit expression of modified channels in cardiomyocytes (38,52,53). The subcellular
Localization and function of CaV1.2 in cardiomyocytes was not affected by fusing APEX2 to a and b (7). Importantly, the Ca\(^{2+}\) currents imparted by these APEX-conjugated Ca\(^{2+}\) channels were stimulated by \(\beta\)-adrenergic agonists, implying that conjugating APEX2 to the channels did not perturb PKA-mediated regulation of this process. Incubating isolated ventricular cardiomyocytes with biotin-phenol for 30 minutes followed by exposure to hydrogen peroxide for 1 minute induced biotinylation of proteins in a striated Z-disk pattern, consistent with proper localization of Ca\(^{2+}\) channels within the T-tubules of cardiomyocytes (Figures 2C and 2D). Affinity purification of the biotinylated proteins was then performed with the use of streptavidin-sepharose (Figure 3). Via mass spectrometry, we identified nearly all previously reported members of the cardiac CaV1.2 complex, as well as many proteins known to be in close proximity to CaV1.2 (7). A detailed description of sample preparation has been thoroughly reviewed elsewhere and is beyond the scope of this review (2,3,54).

We found that proximity labeling of the Ca\(^{2+}\) channel neighborhood can also be performed in beating hearts, capturing the full effect of extracellular matrix, myocyte-myocyte, and myocyte-nonmyocyte interactions, the importance of which has become increasingly recognized over time (55,56). For these experiments, Langendorff-perfused hearts were retrograde perfused with Krebs solution for 10 min before the addition of biotin-phenol for 15 min (Figure 2C). Electrocardiograms were monitored throughout the infusion protocol to ensure viability of the preparation. Streptavidin-fluorescein isothiocyanate immunofluorescence of tissue sections of Langendorff-perfused hearts also showed biotinylation of proteins in a striated Z-disk pattern (7).
In our APEX2-Cav1.2 experiment, we also labeled nuclear, mitochondrial, ribosomal, ER, and Golgi proteins (7). This is because $\alpha_{1C}$ and $\beta_{2B}$ APEX fusion proteins not only exist in the transverse tubule, but rather are constantly being created at the ribosomes and trafficked there from the rough ER. Wherever there are transgenic channels in the cell, nearby proteins are biotinylated. Moreover, it is challenging to differentiate direct physical interactors from bystanders even within the subcellular area of interest. Suspecting that the transducer of adrenergic regulation of Ca2$^+$ channels would alter its interaction with Cav1.2 after exposure to $\beta$-adrenergic agonist, whereas most bystander proteins would not, we compared the Cav1.2 neighborhood in the absence and presence of isoproterenol in isolated cardiomyocytes and Langendorff-perfused beating hearts, similarly to studies using heterologously expressed G-protein-coupled receptors (20,21).

We preincubated isolated cardiomyocytes from $\alpha_{1C}$-APEX and $\beta_{2B}$-APEX mice with biotin-phenol for 30 min, and during the final 10 min the cells were also exposed to either isoproterenol or vehicle (Figure 2D). The biotinylated proteins were purified and quantified with the use of tandem mass tag triple-stage mass spectrometry (TMT SPS MS$^3$) (Figure 3). We also probed the effect of isoproterenol using Langendorff-perfused whole hearts. We were careful only to include in our analysis hearts with demonstrable adrenergic activation, assessed with the use of electrocardiography and phosphorylation of phospho-lamban. The protein quantification changed for several proteins: We detected an isoproterenol-induced recruitment of the PKA catalytic subunit to Cav1.2 and a 30% to 50% decrease in the amount of Cav1.2 after exposure to $\beta$-adrenergic agonist, whereas most bystander proteins would not, we compared the Cav1.2 neighborhood in the absence and presence of isoproterenol in isolated cardiomyocytes and Langendorff-perfused beating hearts, similarly to studies using heterologously expressed G-protein-coupled receptors (20,21).

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In another study, BioID was coupled to the structural protein titin (Figure 4) (71). Rudolph et al. (71) generated knock-in mice with BioID spliced into titin exon 28, corresponding to the location of the Z disk-1.
band transition. Mice were continuously supplied with supplemental biotin dissolved in their water. The authors compared this subdomain of the titin proteome, described as the sarcomeric proteome, in quadriceps and heart, finding 100 proteins in common (81% of labeled proteins in the heart and 41% in skeletal muscle). They reported that 5% of isolated protein was biotin labeled and, using titin’s immunoglobulin-like domains as reference points, calculated that the BioID labeling radius was 7-15 nm. As in the Jph2-BioID knock-in, no untoward phenotypic or functional effects of the knock-in were noted.

In another study, BioID was fused to N-cadherin and was expressed in neonatal murine cardiomyocytes using an adenovirus (Figure 4) (72). The N-cadherin–BioID fusion localized to the cell-cell contacts, as would be expected, and after biotin was added to the cell culture for 24 hours, proteins along the cell-cell contacts and Z disks were labeled. Mass spectrometry identified 917 proteins, which after setting a threshold fold change of >10 and p < 0.001 was reduced to a final list of 365 proteins, comprising primarily adaptor and adhesion proteins that promote junction specialization (72).

The BioID methodology has also been applied to elucidating the interactome of heterologously expressed wild-type Kir2.1 and a trafficking-deficient type 1 Andersen-Tawil syndrome mutant Kir2.1314-315 channel in HEK cells (73). Kir2.1 is an inward rectifying K⁺ current, playing important roles in setting the membrane potential and controlling excitation and action potential duration of ventricular cardiomyocytes (74). Mass spectrometry identification of avidin-purified biotinylated proteins revealed a total of 218 high-confidence Kir2.1 neighbors, including 75 proteins that were preferentially proximate with wild-type channels, 66 proteins that were preferentially proximate with the mutant Kir2.1 channels, and 77 proteins that were proximate with both wild-type and mutant Kir2.1 channels (73). Based on these BioID-identified interaction studies (73), the authors concluded that the pathogenesis associated with the Kir2.1314-315 mutation involves malfunctions in the mechanisms governing Kir2.1 trafficking from the Golgi to the cytoplasmic membrane, in agreement with prior studies (75-77).

Several desmosome-associated proteins, including plakophilin-4 (PKP4) were found to interact with both wild-type and Kir2.1314-315 channels in HEK cells. Kir2.1 and PKP4 colocalize at intercalated disks and Z disks in cardiomyocytes, and PKP4 is a positive regulator of Kir2.1 current density when coexpressed in HEK cells (73). Taking these findings together, this study suggested that proximity labeling may reveal how and why specific mutations affect the trafficking and function of ion channels.

**COMPARISON OF BioID AND APEX LABELING METHODS**

Whereas we identified more than 3,800 proteins and multiple peptides for over 3,000 proteins using APEX2-conjugated Ca²⁺ channels (7), Feng et al. (70) identified 550 proteins with at least 2 unique peptides. These differences speak to the promiscuity of APEX-catalyzed biotin-phenoxyl radicals diffusing out to sterically available or “exposed” tyrosines, relative to the BioID approach where the proximity-labeling enzyme itself must directly tag nearby basic residues, such as lysine. The labeling substrate in APEX experiments is biotin-tyramide, which can diffuse easily across membranes, although the radicals are membrane impermeable. The available substrate for BioID labeling depends on the vehicle, route of administration, and pharmacokinetics in the model system. Biotin is a carboxylic acid and is less membrane permeable than biotin-tyramide. Our experimental approach made use of mice with doxycycline-inducible cardiac-specific over-expression of the labeling enzyme (7). Feng et al. (70) instead used a knock-in approach with the native gene dosage of their gene of interest. As noted before, APEX experiments permit the characterization of rapid subcellular changes during signaling events and it is feasible to label in a Langendorff preparation ex vivo, whereas BioID can take several days to fully label the proteome subdomain. On the other hand, the BioID approach should not alter reactive oxygen species signaling that may occur in APEX-labeled cells, although any changes related to exposure to hydrogen peroxide in APEX labeling would likely be minimal because they would have to occur during the 1-minute labeling interval before quenching of the labeling reaction.

**USE OF HORSERADISH PEROXIDASE-BASED PROXIMITY LABELING**

Horse radish peroxidase (HRP) is inactive in a reducing environment such as the cytosol of a cell (78). HRP is active in the oxidizing secretory pathway (Golgi and ER lumen) and extracellular surface, and catalyzes the same labeling chemistry as APEX2 with biotin-phenol (19,79). HRP was fused to the extracellular region of the junctional adhesion molecule C (JAM-C), designed to define the receptors localized with JAM-C at the cell junction and to determine which ones co-traffic with JAM-C in endothelial cells (80). Transfected human umbilical vein endothelial
cells were exposed to biotin-phenol for 30 min, and proteins neighboring JAM-C were biotinylated during a 1-minute incubation with hydrogen peroxide. To specifically identify neighboring proteins within intracellular stores of JAM-C, ascorbate, a membra-ne-impermeant inhibitor of HRP, was used to block the proximity-labeling reaction at the extracellular side of the membrane. In addition, APEX2 was conjugated to the intracellular domain of wild-type JAM-C or a mutant JAM-C with 4 arginine substitutions for ubiquitinated lysine residues. Using these multiple proximity-labeling approaches, the authors concluded that JAM-C cotraffics with receptors associated with changes in permeability, such as vascular endothelial cadherin, neuropilin (NRP) 1, NRP-2, and some integrin subunits, but not with junctional proteins associated with transmigration of leukocytes, such as the lateral border recycling compartment components (80). This study demonstrated the full utilization of proximity labeling to explore both extracellular and intracellular neighborhoods of a protein of interest as well as the trafficking of the protein.

NEXT-GENERATION PROXIMITY LABELING

Recent innovations in proximity labeling, primarily complementation, can help to address the issue of “false positives.” Han et al. identified 24 potential “cut sites” in the wild-type APEX (5). These 24 pairs were conjugated with FK506-binding protein (FKBP) and FKBP-rapamycin-binding domain to permit rapamycin-induced complementation. Ultimately, the 200 amino acid N-terminal fragment pairs and 50 amino acid C-terminal pairs were found to have the most favorable characteristics and were referred to as “AP” and “EX,” respectively (Figure 1C). These underwent multiple rounds of positive and negative selection with yeast display-based directed evolution to increase the strength of labeling and to ensure that labeling occurs only with complementation. Han et al. (5), with the use of rapamycin induction, were able to label the proteome of the mitochondrial-ER interface, and through fusing AP and EX to ribonucleoproteins specific to a particular noncoding RNA, label the proteome of this particular RNA. Although several split BioIDs have been developed, a faster split TurboID, developed and validated in the same manner as split APEX, is now available, allowing for rapid in vivo proximity labeling with high spatial specificity (81).

CONCLUSIONS

Proximity proteomics, an avenue of investigation developed only in the past 10 years, holds great promise for advancing our understanding of cardiovascular biology. As these methods continue to mature, several concepts should be kept in mind to ensure high-quality work and worthwhile investment in time and resources. A worthwhile question might be, “What will we do with these results?” Situations well suited to these methods may involve suspected though missing interacting elements that have avoided detection, and after labeling identification can be evaluated in some manner. We found that having a functional or biochemical readout to confirm the effect of our perturbation (β-adrenergic stimulation)—heart rate elevation and phospholamban phosphorylation in our case—allowed us to cull untreated samples and increase the sensitivity of the method to detect changes in signaling. Examining the pathological alterations of the proteome in vivo lends itself particularly well to biotin ligase labeling, whereas more acute changes, such as signal transduction, may favor APEX labeling.

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