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Visualizing protein partnerships in living cells and organisms

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In recent years, scientists have expanded their focus from cataloging genes to characterizing the multiple states of their translated products. One anticipated result is a dynamic map of the protein association networks and activities that occur within the cellular environment. While *in vitro*-derived network maps can illustrate *which* of a multitude of possible protein–protein associations could exist, they supply a falsely static picture lacking the subtleties of subcellular location (*where*) or cellular state (*when*). Generating protein association network maps that are informed by both subcellular location and cell state requires novel approaches that accurately characterize the state of protein associations in living cells and provide precise spatiotemporal resolution. In this review, we highlight recent advances in visualizing protein associations and networks under increasingly native conditions. These advances include second generation protein complementation assays (PCAs), chemical and photo-crosslinking techniques, and proximity-induced ligation approaches. The advances described focus on background reduction, signal optimization, rapid and reversible reporter assembly, decreased cytotoxicity, and minimal functional perturbation. Key breakthroughs have addressed many challenges and should expand the repertoire of tools useful for generating maps of protein interactions resolved in both time and space.

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Introduction

In recent years, scientists have expanded their focus from cataloging genes to characterizing the multiple states of their translated products. This endeavor will provide a dynamic map of the protein association networks and activities that occur within the cellular environment. While *in vitro* studies are often used to determine the identities and characterize the energetic and structural features of novel binding partners, these studies take place outside of a cellular context. That this perturbation may lead to artifacts not reflective of the native cell state provides primary motivation for the development of novel approaches for probing protein interactions in live cells. A second motivation stems from recent findings that intracellular signaling networks can be spatially regulated, leading to localized activation [1,2]. Destroying the cellular architecture erases this spatial information, blinding researchers to the complexities of the systems under study. While the use of *in vitro*-derived network maps to describe signaling pathways can illustrate *which* of a multitude of possible protein–protein associations could exist, they supply a falsely static picture lacking the subtleties of subcellular location (*where*) or cellular state (*when*). Generating protein network maps informed by subcellular location and cell state requires novel approaches that both accurately characterize the state of protein associations in living cells and provide precise spatiotemporal resolution.

In this review, we highlight recent advances in visualizing protein interactions under increasingly native conditions. These developments discussed include second generation protein complementation assays (PCAs), chemical and photo-crosslinking techniques, and proximity-induced ligation approaches. These developments have focused on background reduction, signal optimization, rapid and reversible reporter assembly, decreased cytotoxicity, and minimal functional perturbation. Key breakthroughs have addressed many challenges and should expand the repertoire of tools useful for generating maps of protein interactions resolved in time and space.

Beyond traditional FRET

Historically, affinity chromatography and co-immunoprecipitation techniques [3,4] have been used widely to isolate protein complexes from cell lysate for subsequent analysis by mass spectrometry or Western blotting. Two techniques that began to move the analysis of protein interactions into cells are yeast two-hybrid screens and Förster resonance energy transfer (FRET). There exist many excellent and recent reviews on these techniques [5–8], and we do not discuss either of them in detail. However, two recent developments that address certain

limitations of traditional FRET-based assays deserve mention.

First, a report by Lee *et al.* [9] describes a unique co-translocation assay for visualizing protein interactions and their inhibitors in live cells. The authors exploit the ability of protein kinase C delta (PKCδ) to translocate to the plasma membrane from the cytosol in response to specific stimuli. They fused PKCδ to a fluorophore-tagged 'bait' protein and identified interaction partners by virtue of their co-translocation to the plasma membrane with a fluorescently tagged protein target (as visualized by fluorophore colocalization). It was suggested that this assay could identify putative inhibitors of a dual protein association, as such molecules would block co-translocation. This technique requires less demanding construct design than FRET-based assays, which rely upon extensive controls and optimization of fluorophore signal and orientation. The approach also shows potential utility for high-throughput screening. It must be noted, however, that the assay suffers from the need to fuse each member of the protein partnership to another protein (PKCδ and/or a fluorescent reporter) and is not capable of temporal resolution.

Second, a newly developed time-resolved lanthanide-based resonance-energy transfer method (TR-LRET) monitors the energy transfer between a dihydroreductase (DHFR)–terbium complex and GFP, each of which are fused to proteins of interest and produce a FRET signal upon their interaction [10]. This improvement on traditional FRET exhibits time-resolved imaging and significantly higher signal-to-noise ratios than typical FRET pairs due to the uniquely long fluorescence lifetimes of lanthanides. While this method also suffers from the fusion of large molecules to the target proteins, both examples, despite their drawbacks, represent significant improvements in signal strength.

Protein complementation improved

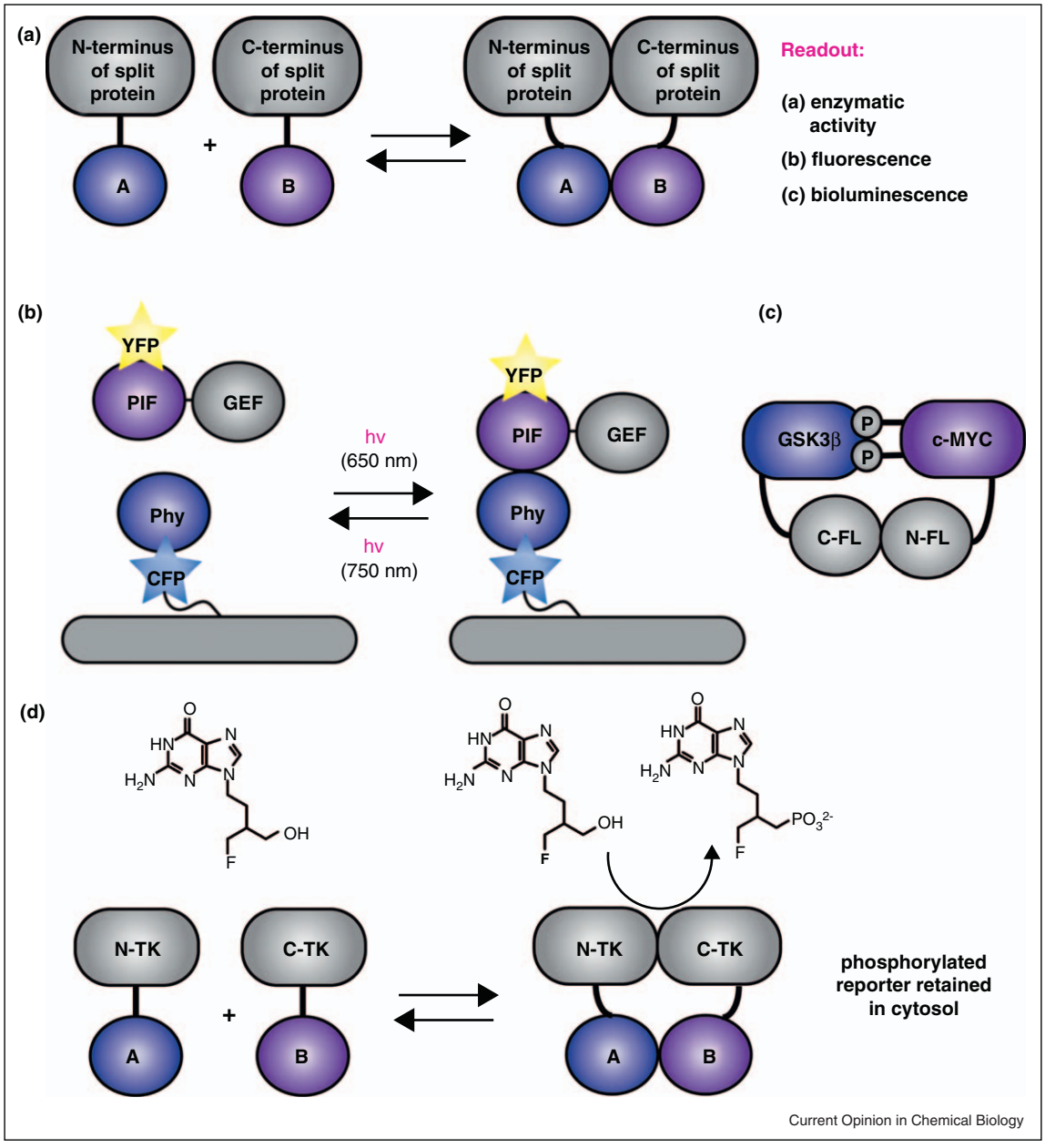
PCAs are used widely to study protein partnerships *in vitro* and in living cells. These assays all derive from the 50-year-old observation of Richards [11] that the single chain protein ribonuclease A spontaneously self-assembles into a native, functional structure even when the chain is split into a pair of polypeptide parts. Subsequent experimentation revealed that the stability of such 'split' proteins varies widely. Thus, as articulated first by Johnsson and Varshavsky [12], if the split protein or enzyme is only marginally stable and provides a fluorescent or functional read-out when reassembled, then fusing the two parts to a pair of potentially interacting proteins provides a tool to monitor and detect the potential interaction. Association of the two interacting proteins increases the effective concentration of the split parts and promotes a native fold, resulting in a fluorescent, bioluminescent, or enzymatic signal. Early PCAs employed the proteins ubiquitin [12], dihydrofolate reductase [13,14] and β-lactamase [15], among others, and were leveraged to identify, characterize and/or exploit dual protein partnerships.

PCAs that monitor the reconstitution of an autofluorescent protein are known as bimolecular fluorescence complementation (BiFC) assays [16–18] and were first reported in 2000 [16]. BiFC assays facilitate the direct monitoring of protein interactions by microscopy, as the intensity of the fluorescent signal resulting from a specific interaction correlates under certain conditions with the strength of the interaction. One challenge in the application of BiFC is that the reconstitution of a fluorescent protein can be slow, as it requires both protein folding and covalent chemistry, and these slow kinetics limit temporal resolution. Recognizing this challenge, Kodama and Hu [19] developed an enhanced YFP variant that is fourfold brighter and matures more rapidly than Venus, an enhanced GFP variant. These improvements are important because enhanced brightness decreases illumination requirements, reducing fluorophore bleaching and phototoxicity. Further protein engineering will be required to address remaining concerns, namely that these proteins are large and tend to oligomerize, and that the overexpression of fusion proteins can also impact native cellular activities.

Work reported this year demonstrated that light can control macromolecular associations as well as report on their formation. Voigt and Lim [20•] reported an engineered version of phytochrome B (Phy) that associates with the PIF domain upon irradiation with 650 nm light (Figure 1b). Exposure of Phy to red light (650 nm) directly induces chromophore photoisomerization and results in a conformational change; the PIF domain specifically associates with the 650 nm-irradiated Phy state. This interaction localizes guanine nucleotide exchange factors to the cell membrane and enables downstream actin polymerization. The recruitment is rapid and reversible upon irradiation with 750 nm (infrared) light. Because it exhibits such high spatiotemporal control, this method could be used to control protein interactions within a variety of cellular environments. Looking to the future, the approach would benefit from the development of several visually orthogonal light-controlled domains, as their combination would allow for the simultaneous control of multiple protein interaction pairs. Additionally, the development of smaller protein tags would enhance the applicability of this method, as visualization now relies on fusions with large fluorescent proteins.

A variation on traditional BiFC makes use of split versions of the bioluminescent enzymes *Renilla* and firefly luciferase. Unlike fluorescent proteins, luciferases are not subject to fluorophore maturation delays and they sidestep phototoxicity and photobleaching concerns. As a result, they can monitor protein interaction pairs with greater temporal precision than reporters providing fluorescent

Figure 1



Split reporters improved. **(a)** Schematic diagram of the split reporter approach to detect dual protein interactions. **(b)** Strategy for reversible light-controlled induction of the PIF-Phy interaction. **(c)** Split firefly luciferase enables visualization of the phosphorylation-mediated GSK3β–cMyc interaction. **(d)** Rationale for PET-based imaging by split thymidine kinase. Interacting fusion proteins reconstitute thymidine kinase, which phosphorylates radioactive PET probe [18F]-FHBG.

readouts. The bioluminescence reaction does, however, require the addition of a luciferin or coelenterazine substrate; these small molecules are oxidized to oxyluciferin or coelenteramide with the concomitant production of light. Several advantages of split luminescence assays were highlighted in a recent report by Ishimoto *et al.* [21]. In this study, N-terminal and C-terminal fragments of firefly luciferase were fused separately to actin monomers, with luciferase activity reconstituted upon polymerization. This assay revealed that UV irradiation led to transient actin upregulation, which the authors propose to be a possible protective mechanism. Use of the split luciferase enabled a dynamic, real-time analysis that would be impossible using the traditional method of rhodamine-phalloidin staining. This approach could potentially be engineered to study a wide range of protein interaction pairs in live cells. A recent report by Hida *et al.* [22] has extended the utility of luciferase complementation to the simultaneous

spatiotemporal tracking of multiple protein complexes. This study reported several new split luciferases possessing novel and unique spectral properties (such as emission wavelengths). Their combination allowed for concurrent monitoring of multiple Smad interactions in a live *Xenopus laevis* embryo. The authors also demonstrated time-lapsed and luminescent imaging of dynamic, reversible protein interactions. While future work would also benefit from continued optimization of emission intensity and wavelength, as well as the pursuit of smaller and more photostable reporters, the ability to monitor multiple species simultaneously is critical for distinguishing multimolecular complexes that share only a subset of components.

Moving protein interaction studies into whole organisms is essential in order to draw connections between the biology in a Petri dish and in higher organisms and humans. Fan-Minogue *et al.* [23•] engineered a split luciferase system to analyze the interaction between glycogen synthase kinase-3β (GSK3β) and cytoplasmic Myc protein (c-Myc), a known oncoprotein (Figure 1c). This report demonstrated that a split sensor could be extended to quantitatively monitor c-Myc activation as well as the potency of interaction inhibitors in live mice. While some nonspecific binding activity will require future optimization, this sensor shows promise for high-throughput screens and rational drug design, as changes in the luminescent signal were shown to indicate inhibitor efficacy more rapidly than alterations in tumor size.

While the majority of split reporter systems utilize fluorescent or bioluminescent readouts, enzymatic activity (such as phosphorylation) can also be harnessed to analyze endogenous protein interaction pairs. A recent study by Massoud *et al.* [24••] describes a unique positron-emission tomography (PET)-based kinase complementation system to visualize and quantify PPIs in live mice. The group designed a split thymidine kinase (TK) reporter system where radiolabeled, cell-delivered TK substrates were phosphorylated by reconstituted TK, enabling their selective retention in cells with PPI-induced TK complementation (Figure 1d). As a proof of principle, the group was able to image the rapamycin-mediated interaction between FRB and FKBP in live mice at a significantly greater tissue depth than bioluminescent imaging. With further engineering, this PET-based split reporter system shows potential for the development of clinical PPI imaging systems in live animals.

Crosslinking becomes sophisticated

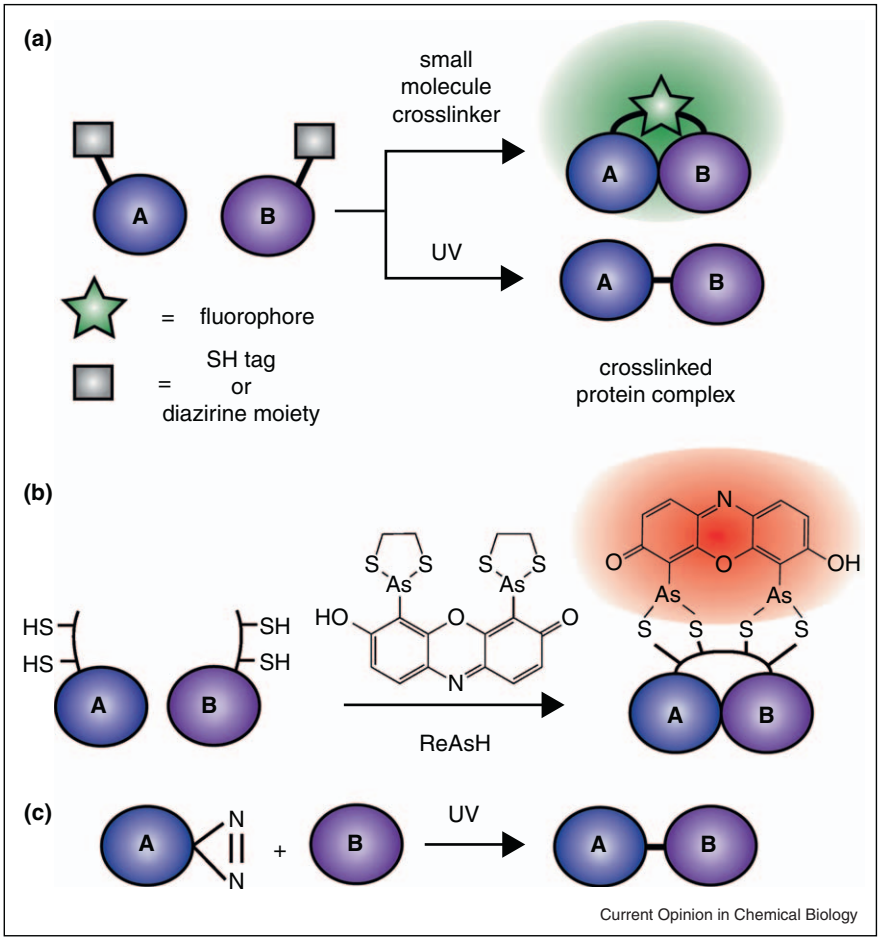
Affinity purification and immunoprecipitation assays are used widely to isolate and identify robust protein interactions, yet they can fail when challenged with transient or unstable complexes. These assays are also subject to the caveats associated with performing experiments in lysate. Performing the cross-linking reaction in live cells overcomes these challenges (Figure 2a). After cross-linking, standard techniques such as SDS-PAGE, Western blotting, mass spectrometry, or microscopy can be used to analyze the stabilized complexes [25].

Bis-arsenical dyes such as FlAsH and ReAsH direct the crosslinking reagent to a pre-determined protein pair and offer the added benefit of a turn-on fluorescent signal for visualizing the protein interaction in live cells. Introduced in 1998 by Tsien and coworkers [26], bis-arsenicals are derivatives of fluorophores that have been modified with two arsenic (III) substituents, usually in the form of ethanedithiol chelates. For reasons that are not understood [27], bis-arsenicals chelated by two ethanedithiol molecules are not highly fluorescent. However, upon thiol exchange with a protein containing an appropriate tetracysteine array [26], bright fluorescence is observed. In 2007, Luedtke *et al.* reported [28] that bis-arsenical binding and fluorescence could occur even when the array of four cysteine side chains was split between two protein partners. This advance, termed bipartite tetracysteine display [28,29], enables the direct visualization of protein interaction pairs (Figure 2b). Bipartite tetracysteine display has been recently used to investigate the amyloid-β aggregation pathway *in vitro* [30•]. Amyloid formation is hypothesized to proceed from monomer to fibril *via* an oligomer state that is notoriously difficult to visualize. The authors engineered amyloid-β monomers that could bind split FlAsH both as oligomeric and fibrillar aggregates. The combination of kinetic data from split FlAsH and thioflavin T fluorescence enabled differentiation of the two states. These results provided key support for the mechanism of amyloid-β fibril formation. This split FlAsH-based approach shows great promise for the kinetic characterization of other aggregating (non-disulfide containing) proteins.

The ability of bis-arsenicals such as FlAsH and ReAsH to selectively bind protein interaction pairs led Dexter and Schepartz to develop a strategy to selectively visualize these complexes using electron microscopy (EM) [31]. This strategy exploits the observation that irradiation of ReAsH generates singlet oxygen; if the ReAsH is bound selectively to a protein complex, irradiation in the presence of diaminobenzidine (DAB) produces an opaque DAB polymer that acts as a contrast marker for EM upon treatment with osmium tetroxide. In contrast to antibody-based EM protocols, the DAB polymer signal can be generated in live cells, providing a potentially more realistic picture of the spatial location of the protein complex.

Photo-crosslinking is another rapidly expanding approach for stabilizing and isolating elusive PPIs. For a set of photoreactive moieties (i.e. azide and benzophenone derivatives), UV irradiation results in photo-crosslinking that is highly selective for proximal binding partners due to the extremely short half-life of the reactive intermediates [32]. However, the challenges

Figure 2



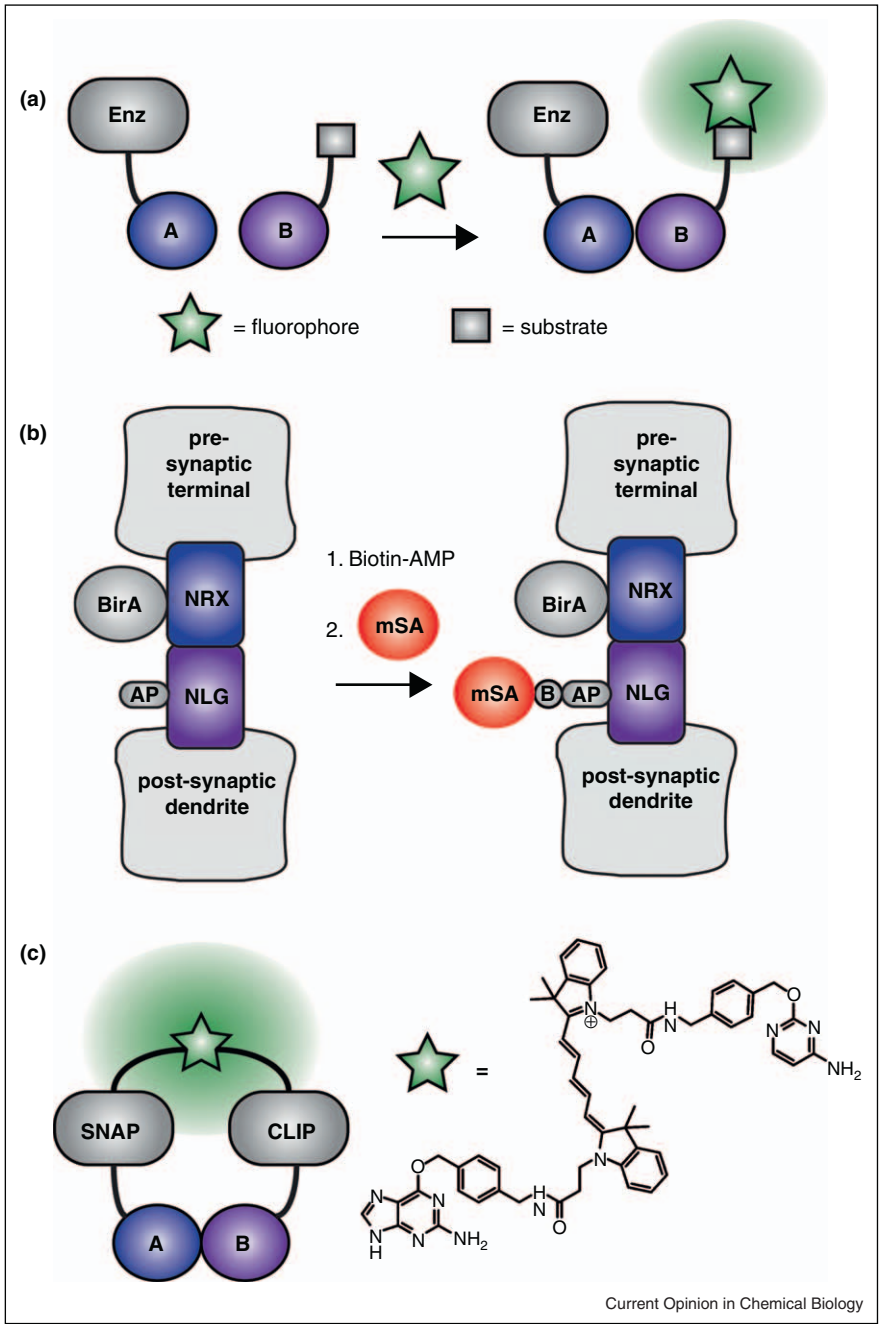
Crosslinking becomes sophisticated. **(a)** Rationale for chemical and photo-crosslinking strategies. Interacting proteins containing specific tags are crosslinked with a fluorescent reporter (top) or photo-crosslinked (below) upon UV irradiation. **(b)** In bipartite tetracysteine display, a pair of CysCys tags are approximated upon intramolecular or intermolecular protein association and bind bis-arsenicals such as ReAsH. **(c)** When applied with engineered tRNA synthetase machinery, diazirine lysine analogs enable UV-induced crosslinking of proximal biomolecules for complex stabilization and further structural analysis.

associated with incorporating these photoactivatable moieties into proteins within a living cell have limited the application of this approach. In response, recent work has pursued the manipulation of orthogonal tRNA machinery to integrate photoreactive amino acid analogs into protein targets (Figure 2c). Chou *et al.* [33] recently described the synthesis of a diazirine lysine analog as a substrate for amber codon-induced pyrrolysyl tRNA machinery; the diazirine moiety was shown to photo-crosslink glutathione S-transferase (GST) monomers in *Escherichia coli* cells. Additionally, Ai *et al.* [34] have used directed evolution to increase the efficiency of diazirine lysine analog incorporation by the pyrrolysyl tRNA machinery. These studies are poised for progress following recent advances made by the Chin lab in orthogonal tRNA design [35] and application of amber suppression technologies into mammalian cells [36].

Proximity-induced ligation: the benefit of being close

One versatile approach toward mapping endogenous dual protein interactions exploits the interaction itself to increase the proximity and reactivity of a bond-forming enzyme with its substrate; this strategy is referred to as proximity-induced ligation. An enzyme capable of transferring tags such as biotin or a fluorophore to a proximal substrate is fused to one interacting partner; the other partner is fused to the substrate. If the partnership assembles, the enzyme is brought into proximity with its substrate and a labeling reaction occurs [37]. The first proximity-induced ligation reactions were reported by Keppler *et al.* [38], who exploited human O_6-alkylguanine transferase (hAGT) to transfer alkyl groups from O_6-benzylguanine derivatives to a proximal cysteine thiol. The AGT labeling method has since been refined into

Figure 3



The benefit of being close. **(A)** General strategy for a proximity-induced ligation. Two proteins of interest (A,B) are fused to a modifying enzyme (Enz) or its substrate. Interacting proteins enable labeling of the substrate tag by the proximal enzyme. **(B)** Detecting neurexin1β (NRX)-neuroligin1 (NLG) interactions in live neurons with biotin ligase (BirA). Interaction of NRX and NLG increases the proximity of BirA to its acceptor peptide (AP) and biotin (B) transfer occurs. AlexaFluor-labeled monovalent streptavidin (mSA) then binds the ligated biotin, enabling direct visualization of the protein interaction. **(C)** In S-CROSS, bifunctional molecules consisting of a cyanine dye modified with two SNAP-tag or CLIP-tag substrates crosslink interacting proteins A and B to visualize their interaction in cells. (A Cy5 dye modified with both a SNAP-tag and CLIP-tag substrate is shown.)

the widely used tools SNAP-tag [38] and CLIP-tag [39]. The related Halo-tag method developed by Promega exploits a modified haloalkane dehalogenase (DhaA) enzyme [40]. The advantages of these proximity-induced ligation systems include small enzyme sizes (at least relative to GFP) and significant flexibility in the identity of the ligated molecule.

Dual protein interactions can also be mapped using a proximity-induced ligation method developed by Ting

and coworkers [41]. In this case, the enzyme fused to one protein partner is *E. coli* biotin ligase (BirA); the other partner is fused to a short acceptor peptide (AP) substrate. Recent developments include a pair of engineered *E. coli* lipoic acid ligases (LplA) capable of attaching 7-hydroxycoumarin [42] or Pacific Blue [43] fluorophores to the transposable LplA acceptor peptide (LAP) on a fusion protein. These methods enable precise intracellular labeling and superresolution imaging [44]. Experiments that combine visually orthogonal enzyme–substrate pairs also allow multicolor labeling.

Recent work extended the scope of proximity-induced ligation methods for protein visualization in two interesting ways. In one report, a method referred to as BLINC ('biotin labeling of intercellular contacts'), BirA-AP ligation was used to identify trans-synaptic associations between neurexin1β (NRX) and neuroligin1 (NLG) [45••] (Figure 3b). Thyagarajan *et al.* expressed BirA-NRX and AP-NLG fusion proteins in live neurons; upon their association, the BirA-NRX fusion catalyzed the biotinylation of the proximal AP-NLG substrate (on an adjacent cell). Biotinylation was subsequently visualized by staining with fluorophore-modified monovalent streptavidin. BLINC enables improved spatial resolution and more rapid protein complex visualization than GFP-based approaches, as it does not require a slow fluorophore maturation step and is not hampered by high background due to fragment self-assembly. BLINC can additionally be coupled with pulse-chase assays or a reversible label for greater temporal resolution. However, BLINC labeling is irreversible, and mSA staining requires an extensive washing process.

A second extension of the proximity-induced ligation theme combines several available strategies in a process called S-CROSS [46•]. This modification involves a fluorescent cyanine dye conjugated to two substrates for SNAP-tag or CLIP-tag or both (Figure 3c). The spatial approximation of the SNAP-tag (or CLIP-tag) and substrate ligates the bivalent dye to a first target protein. The second reactive moiety is then capable of simultaneously crosslinking the interacting proteins. Because the two reactive handles are bridged by a fluorophore, this method visualizes the protein complex. While at this point the method functions only in cell extract, it can also be effectively paired with *in vivo* fluorescence microscopy to correlate observed colocalization and direct protein interactions.

Conclusion

Over the past several years, varying approaches to the visualization and tracking of PPIs have seen significant advancements. Many of these techniques rely on fusion proteins, which tend to be large and can perturb native function. A similar concern exists when using enzymatic domains as in the case of proximity-induced ligation, unfolded portions of protein domains as seen in PCAs, or even substrate APs. Thus careful analysis of fusion constructs is always required. Nonetheless, these techniques are blossoming into increasingly useful and interesting tools that have the potential to reveal previously obscured biological phenomena. In the case of fluorescent reporters, future optimization will focus on efficient chromophore maturation and reduced self-association. Increased signal intensity may be a product of high-throughput library screening to enhance the current repertoire of cross-linkable amino acid analogs or molecular sensors. Additionally, precise temporal control of PPI tracking will greatly benefit from developments involving reversible and photoswitchable labels, coupled fusion sensors for monitoring multiple interactions, and tag specialization for targeted intracellular delivery. These developments will enable increasingly refined spatiotemporal mapping of PPIs in their endogenous cellular environments. Concerted applications combining enzymatic specificity and synthetic versatility will continue to spearhead our growing understanding of protein signaling networks in living cells.

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- • of special interest
- •• of outstanding interest

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