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Dynamics of protein complex components

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Identifying protein-protein interactions (PPIs) is necessary to understand the molecular mechanisms behind cellular processes. This task is complicated by the facts that many proteins can interact simultaneously (i.e. a protein complex) and may participate in more than one distinct complex. Because of this, a large number of combinatorial arrangements are possible, both of PPIs and complexes, making it a difficult task to identify all truly interacting proteins. Protein interactions also range from stable to highly transient assemblies, with lifetimes on the order of seconds [1]. Therefore, studies identifying PPIs must not only contend with the arrangement of proteins into PPIs and complexes, but the stability of the interactions as well. Because of the difficulty of the task, many approaches have been used to identify and study the dynamics of PPIs. In this review, we will summarize a number of the techniques currently used to identify protein-protein interactions, with a focus on recent developments.

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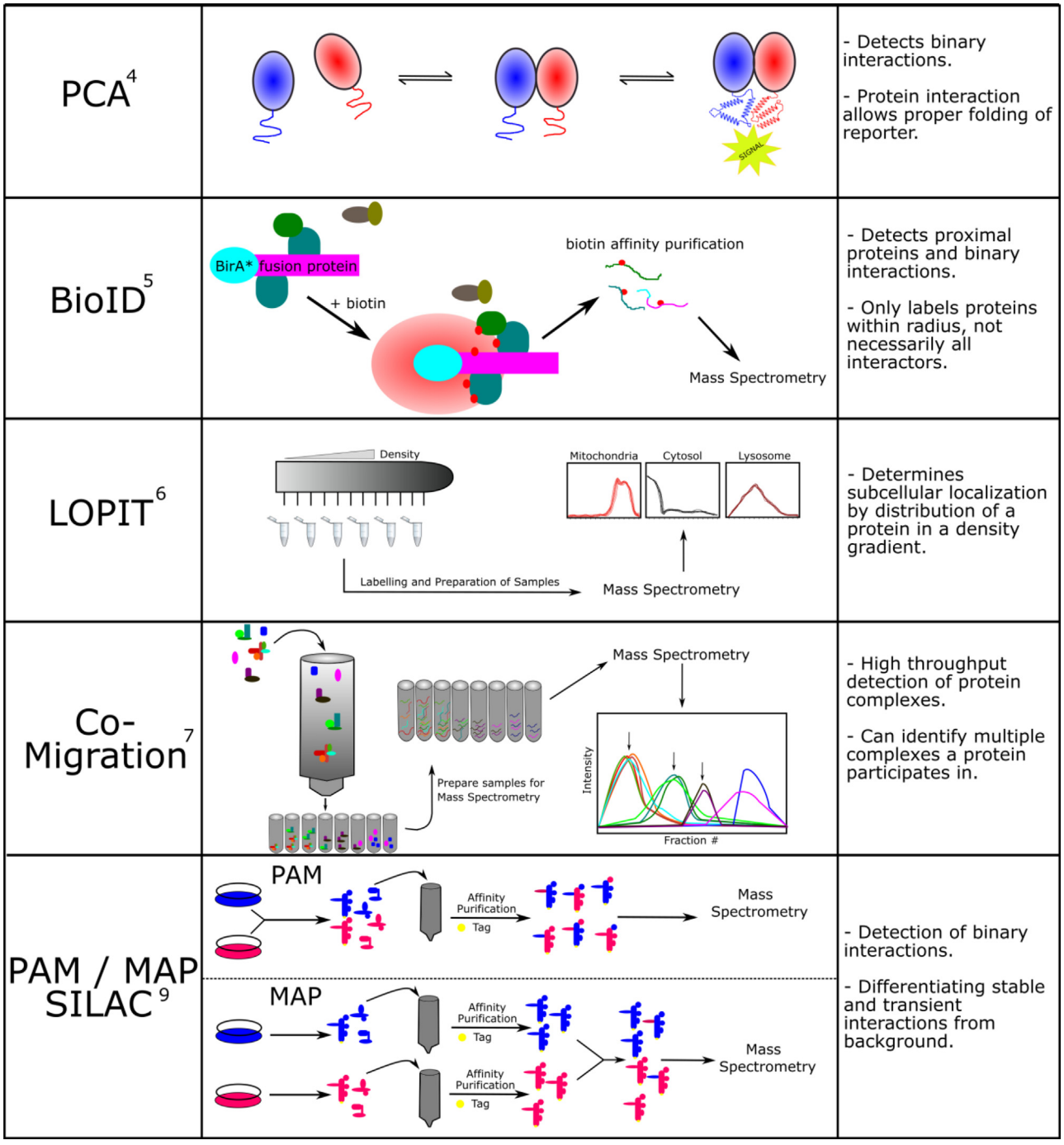
Introduction

Protein-protein interactions (PPIs) underlie the majority of processes performed within and between cells. Proteins of all types form homogenous or heterogeneous complexes via noncovalent interactions with one or more partners, constituting the interactome of the cell. These protein complexes are necessary to perform the biochemical reactions required to maintain cellular function. Therefore, understanding protein interactions — their assembly, and stoichiometry — is necessary to elucidate the mechanisms underlying cellular functions. Recent publications, such as the work on miRNA-mediated gene silencing pathways [2••], and on nuclear envelope kinases [3] demonstrate the utility of these approaches. Many experimental approaches have been developed to study the composition and dynamics of PPIs, including using reporters to detect direct interactions [4]; detecting proximal proteins by biotinylation [5]; measuring the degree to which proteins co-fractionate across density gradients or chromatographic separation [6,7•,8] and measuring the dynamic exchange of labelled subunits [9,10•]. Some of these approaches are based on affinity purification and yeast 2-hybrid (Y2H), which are two of the most well established techniques for PPI detection, and have been the subject of several previous reviews [11–17]. Because of the complexity of the task, these complementary approaches and others are likely necessary to fully uncover an organism's interactome. In this review we will discuss some of the major approaches to detecting and monitoring PPIs, focusing on some of the newer approaches.

Protein complementation assay

Protein Complementation Assay (PCA) is a technique where a reporter protein, broken into two polypeptides, has one segment attached to a target protein, and the other segment to a bait protein [4]. This approach is an improvement on yeast 2-hybrid (Y2H), replacing the use of a split transcription factor to generate the reporter signal with a specially designed split reporter protein [18]. The split reporter is designed so that when an interaction occurs between the target and bait proteins, the reporter polypeptides are in close enough proximity to fold into the functional reporter protein and produce a detectable signal (Figure 1). This is a key advantage of PCA over Y2H methods, in that the two polypeptides of the reporter protein don't form separately foldable domains. The two subunits are chosen such that they only fold once brought together by the direct interaction of target and bait proteins (Figure 1). This prevents already folded domains of the reporter generating signal when there is no interaction from the target proteins, improving the confidence of the interactions [19]. Another differentiating factor is that Y2H techniques often involve an artificial localization to the nucleus, whereas PCA does not require a specific subcellular localization [20]. Two of the more common reporter proteins are murine dihydrofolate reductase (DHFR), and firefly luciferase. However, there are many possible variants for the readout in PCA, including dominant-selection, colorimetric, luminescence, or fluorescence detection [19]. Recent iterations of PCA include a smaller enzyme for luminescence based PCA's: Verhoef *et al.* [21] report a nanoluciferase (NanoLuc) from deep sea shrimp (*Oplophorus gracilirostris*), which is one third the size of firefly luciferase, potentially reducing issues with the tag interfering with folding or subcellular targeting of the bait

Figure 1



Cartoons depicting the different approaches for probing protein-protein interactions. Protein Complementation Assay (PCA): target and bait proteins fused to different, unfolded halves of the reporter protein come into physical contact. The protein-protein interaction allows the reporter protein to correctly fold and produce signal. Proximity-dependent biotin identification (BioID): a target protein is fused to BirA. In presence of biotin, BirA produces reactive biotinoyl-AMP, which biotinylates proximal proteins. Localization of Organelle Proteins by Isotope Tagging (LOPIT): Organelles are separated by a density gradient. Fractions from the gradient are labelled and prepared for mass spectrometry. Distributions of proteins within the gradient are used to assign subcellular localization. Co-migration: Proteins are separated on a gradient into fractions. Similar chromatographic profiles suggest proteins migrated together in a complex. Purification After Mixing/ Mixing After Purification (PAM/MAP) - SILAC: Labelled cell lysates are either mixed then purified, or purified then mixed. The labelled protein complexes will exchange subunits to greater degrees depending on how long they have been mixed.

or prey. NanoLuc also has a higher activity and different substrate than firefly luciferase, allowing the two to be multiplexed without the need for special filters and deconvolution of signal that previous multiplexing experiments required [21,22].

Proximity-dependent biotin identification

Proximity-dependent biotin identification (BioID) is a technique where a promiscuous biotin ligase is fused to a protein of interest. This fusion protein then biotinylates any proteins it encounters in *vivo* [5] (Figure 1). This was achieved using a mutant of the *Escherichia coli* BirA biotin ligase [23,24], which has an estimated range of 10 nm from its active site [25•]. This technique is advantageous over other high-throughput PPI assays for multiple reasons. First, it works *in vivo*, meaning labelling occurs under normal environmental conditions. Second, labelled proteins are easy to purify via avidin affinity purification, and are purified independent of complexes, allowing stringent wash conditions to reduce non-specific interactors. Third, the technique is applicable to insoluble proteins, which is valuable as membrane proteins have historically been difficult to work with. Finally, BioID has the capability to identify transient or weak protein-protein interactions, which are often not identifiable by other approaches.

One problem with BioID is the potential for the size of the BirA ligase to interfere with subcellular localization of the protein of interest. This problem has been recently addressed by the development of a new smaller BirA ligase from Aquifex aeolicus [26••]. Called BioID2, this new system reduces the impact of ligase size on subcellular targeting, but also requires a reduced amount of biotin, and its proximity range can be modified by the use of flexible linkers [26••]. A key disadvantage of BioID and BioID2 is that they do not necessarily identify interacting proteins, only proximal proteins (Figure 1). Because of the long incubation time, this technique not only detects protein complexes, but also all proteins that passed within the radius of BirA during the incubation. A new development merging PCA and BioID, called Split-BioID [2••], addresses this issue by splitting the BirA into two segments, which refold when brought into proximity and proceed to biotinylate the vicinal proteins.

Localization of organelle proteins by isotope tagging

Localization of Organelle Proteins by Isotope Tagging (LOPIT) is an approach to identify the subcellular localization of proteins. The original version of the technique separated crude membrane fractions by ultracentrifugation on a self-generating iodixanol gradient [6] (Figure 1). The pairwise comparison of the fractions from the gradient is accomplished by labelling with isotope coded affinity tags (ICAT), the lighter density fraction being labelled with the light isotope affinity tag. The pooled samples are then analyzed by mass spectrometry. One of the big advantages of the technique is that subcellular localization can be assigned to proteins without obtaining pure organelle fractions. Instead the distribution of a novel protein across the gradient fractions is compared by principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) to the distribution of proteins of known localization across the gradient. A goodness of fit algorithm is used to score how well a novel protein fits to the model generated from proteins of known localization.

The original technique has since been improved upon by two new approaches. The first technique is hyperplexed LOPIT (hyperLOPIT) [27••], which has improved sample parallelization, and quantitative accuracy. HyperLOPIT replaces ICAT tagging with tandem mass tags (TMT) which allows multiplexing up to 10 samples, this allows a more comprehensive fractionation scheme with higher resolution. The quantitative accuracy of TMT is improved by the development of synchronous precursor selection (SPS) MS3, which reduces the problem of interference by contaminant peptides. The second technique is LOPIT after Differential ultraCentrifugation (LOPIT-DC) [28•], this technique is designed to be a simplified workflow where density gradient centrifugation of crude membranes is replaced with multiple differential centrifugation steps on a whole cell lysate. The advantages of this technique is a reduction in time, cost, and starting material required, however at the cost of decrease in resolution.

Co-migration

Co-migration (or co-fractionation) is an approach by which protein complexes are fractionated under native conditions, commonly by chromatography [7•,8] or electrophoresis [29], followed by mass spectrometry to quantify and correlate protein amount across the fractions. Co-migration relies on co-complexing proteins migrating together during fractionation, and thus having very similar migration profiles (Figure 1). The main benefits of co-migration are that it is high throughput, has no requirement for tagging the proteins of interest, and allows for a single protein to be found in multiple complexes. Soluble complexes are commonly fractionated by size exclusion [7•,30,31] or ion exchange [8] chromatography, but the same principle can be applied to membrane proteins through native polyacrylamide gel electrophoresis [29,30,32]. Importantly, this technique can be enhanced with the addition of stable isotope labelling of amino acids in cell culture (SILAC) to monitor the changes in protein complexes between a control and a perturbed condition [7•], a major advantage over other techniques that are not designed to study changes to PPIs between conditions.

Co-migration studies often yield large, complex datasets, meaning co-migration can present a bioinformatics challenge. Because of the fact that co-migration studies can

measure thousands of migration profiles simultaneously, and each pair of migration profiles is typically assessed, these studies can present millions or tens of millions of potential interactions. To overcome this, it is common to use a reference database of known interactions, such as CORUM [33]. Automated bioinformatics tools exist to analyze co-migration data [34•].

PAM / MAP SILAC

Another set of SILAC-based techniques are the purification after mixing (PAM) and mixing after purification (MAP) protocols. These build on AP-MS, with PAM adding in SILAC-labelled media to identify non-specific interactions, and MAP identifying weak or rapidly exchanging subunits of protein complexes [9,10•]. With PAM SILAC, cells expressing a tagged protein of interest are cultured in heavy isotope labelled media, while the control condition expresses the tag by itself in light labelled media [35]. The samples are lysed, the two differently labelled lysates are mixed together, then purified on an affinity column, and incubated before elution for a series of time points (Figure 1). Stable interactors can be detected by a higher ratio of the heavy-to-light isotopes, as the stability of the interaction means the two isotopes are not rapidly exchanged. Weak interactors, however, will rapidly exchange, resulting in a detectable heavy-to-light ratio of approximately one [9].

This approach is unable to assess rapidly exchanging subunits, as a ratio of one is usually indicative of non-specific binding. To identify rapidly exchanging subunits, MAP-SILAC can be performed after PAM-SILAC. In MAP-SILAC, purification of the protein of interest is performed separately for each of the labelled samples, the resulting elutions mixed together and mass spectrometry is performed (Figure 1). Because there is no co-incubation time on the affinity columns, there is no opportunity for subunit exchange. Thus, the ratio of rapidly exchanging subunits should have a higher heavy-to-light ratio than in the PAM-SILAC experiments. A recent alternative to PAM and MAP SILAC based on mixing non-equal ratios of isotope labelled samples has also been proposed as a method to rule out non-specific interactions arising from affinity purification [36]. Because of the unequal mixing, background signal will have an unequal isotopic ratio, whereas specific interactions will have a 1:1 ratio.

Conclusion

The techniques discussed in this review have overlapping and distinct capabilities in the determination of protein-protein interactions. Generating data from systematic detection of binary interactions to parallel detection of multiple complexes. Because of their different approaches, each technique generates complementary data. Co-migration is capable of generating a large dataset of potential protein complexes from a small number of samples. BioID generates a list of proximal and interacting proteins, yielding insights on the direct and indirect complex partners for a protein of interest, but can also give evidence to subcellular localization, based on the proximal proteins identified. Subcellular localization can be further interrogated by analyzing the distributions generated by LOPIT. PCAs are capable of many parallel assays to gain high confidence pairwise binary interactions. PCAs are also useful for differentiating true PPIs from proximal and false positive PPIs detected by other techniques. With lists of PPIs, PAM and MAP SILAC help identify the stable and transient interactions from a list of interactions captured by snapshot techniques, such as BioID. In conclusion, each approach gathers a distinct, but overlapping data set of PPIs. Taken together, these techniques all contribute to building a more complete picture of the molecular underpinnings of cellular function.

Conflict of interest statement

Nothing declared.

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