CURRENT EXPERIMENTAL METHODS FOR ANALYZING PROTEIN-PROTEIN INTERACTIONS

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ABSTRACT

With the completion of the Human Genome Project, focus has shifted from cataloging genes and proteins to mapping the large sets of interactions that take place among them. Mapping this network is essential, as proteins do not function in isolation, but rather interact among themselves, or with nucleic acids and other small molecules to form molecular machines. These modular machines involve dynamic assemblies of macromolecules and transmit, but also respond to intra- and extracellular signals, in order to ensure cellular adaptability and reproduction. The present review addresses the need for an updated brief description of most commonly used techniques for the characterization of protein-protein interactions, and aims to suggest combinations of experimental methods best adapted for the proteins of interest.

Keywords: protein-protein interactions, protein purification, protein complexes, protein binding affinity.

REZUMAT

Odată cu terminarea proiectului Human Genome, s-a trecut de la simpla catalogare a genelor şi a proteinelor la descrierea vastelor reţele de interacţii care au loc între ele. Cartografierea acestei super-reţele este esenţială, deoarece proteinele nu funcţionează în izolare, ci interacţionează între ele sau cu acizii nucleici, respectiv cu molecule de dimensiuni mici, pentru a forma maşini moleculare. Acestea sunt rezultatul unor asamblări dinamice ale componentelor macromoleculare, fiind răspunzătoare pentru transmiterea de, dar şi raspunsul la semnale intra- şi extracelulare, în scopul asigurării adaptării la mediu şi replicării celulare. Prezenta trecere în revistă a celor mai comune şi actuale metode de caracterizare a interacţiilor între proteine răspunde necesităţii de a folosi combinaţii experimentale cât mai adecvate proteinelor studiate.

Cuvinte-cheie: interacţiuni proteină-proteină, purificare de proteine, complex proteic, afinitate de legare între proteine.

INTRODUCTION

Proteins are the main actors of cellular machinery, responsible for a bewildering array of functions [1]. For regulation purposes, most proteins interact with other protein partners to form transient complexes in vast networks of protein-protein interactions (PPI) that affect metabolism and adaptation to external stimuli [2]. PPI have thus wide-ranging effects, from altering cell surface receptor sensitivity, to modifying kinetic properties of enzymes and down to regulation of gene expression etc. [3]. As the majority of protein functions are mediated by PPI, whenever the function of one partner protein is identified, its functional and pathway assignment is facilitated. The study of PPI has thus become essential for an in-depth understanding of cell structure and function and its set represents the interactome (interactions of proteins in a given cellular proteome) [4]. Moreover, protein interaction measurements have proven essential tools to unravel the molecular bases of diseases [5].

In addition to ongoing experimental focus to create databases for large protein interaction networks [6, 7], efforts have also focused on

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building the curated human interactome, such as Human Protein Reference Database, that catalogs over 40,000 human protein interactions [8]. Within this framework, we stress the importance of analyzing PPI beyond the available crystal structure of the proteins under study, as these provide only a static „snapshot" of the rugged conformational space any protein experiences before, during and after partner binding. Below, we propose an overview of the main experimental techniques employed for studying PPI. We further highlight what we consider to be their key advantages and limitations, and possible means to use these in an orthogonal manner, with the aim of better characterizing and validating PPI *in vivo*.

I. *In vivo* and *in vitro* analyses of PPI

1. Affinity purification and Co-immuno-precipitation

Affinity purification (AP) is a type of chromatography whereby a 'bait' protein (antibody) is covalently linked to a matrix or resin, non-covalently attached by using antibodies, or using a biotin-streptavidin pair present on the surface. Subsequently, a cell lysate or a mixture containing the proteins of interest is passed through the matrix. The proteins that interact with the 'bait' protein attach to it and are retained. The 'bait' proteins can be further eluted by the addition of related competitor ligands, detergents, or by high salt concentrations [9]. Proteins interacting with the 'bait' protein with low-affinity can also be retained in the resin, when present at higher concentrations. Common interactions that are exploited in AP include antibody-antigen, enzyme-substrate/inhibitor/cofactor and ligand-receptor. A similar principle is in place for the co-immunoprecipitation (co-IP) technique whereby an antibody attached to agarose or magnetic-beads is exposed to a cell lysate. The captured protein interacts with its physiological partner(s) and the entire complex can be further separated and identified via mass spectrometry or Western blotting.

To study *in vivo* interactions, the 'bait' protein is expressed as a recombinant protein fused to a tag molecule, whose properties are exploited for its purification. The proteins that interact *in vivo* with the 'bait' are co-isolated simultaneously from the lysate. For example, tandem affinity purification (TAP) is a well established purification technique that allows the study of protein-protein interactions by using a TAP tag fused at the C-terminus of the 'bait' protein. This fusion tag can be introduced in yeasts via plasmids, in order to be translated within the host. TAP tag consists of 3 domains, namely the calmodulin binding peptide at the N-terminal, followed by the TEV protease cleavage site and the protein A at the C-terminal [10]. The relative order of the domains plays an important role in the purification process as the terminal position of protein A binds to immunoglobulin G (IgG), that allows the isolation of the entire complex from the mixture.

Advantages: AP is a robust, high-throughput method when complemented with mass spectrometry. Numerous tags are currently available in terms of host and proteins of interest. The system is applicable for *in vivo* studies in yeast, bacterial and mammalian hosts, depending on the availability of plasmids to express the fusion protein.

Co-immuno precipitation (Co-IP) is a label free, relatively inexpensive approach (does not require specific infrastructure) that demands low sample consumption. Additionally, Co-IP allows the study of proteins in their native configuration. The use of agarose or magnetic beads is an advantage for easier precipitation.

Limitations: AP can lead to false positive results. It is not the best choice to study weak interactions, as very high concentrations of 'bait' proteins are required. The tag bound to the 'bait' protein can potentially obstruct its interaction with the potential partners, or, during purification, the tag may not protrude enough to elute out the protein complex from the mixture, affecting protein expression levels.

Co-IP is highly dependent on the availability of primary antibodies. High-background caused by non-specific interactions due to the presence of a large variety of proteins in the lysate or in the agarose beads can also cause non-specific binding. Due to the harsh elution

conditions, target antigen-antibody complex may be destroyed. Additionally, *in vivo* interactions cannot be studied with Co-IP, whereas transient interactions *in vitro* may be difficult to detect.

2. Yeast two hybrid

Yeast two hybrid **(**Y2H) is a sensitive *in vivo* detection method of PPI, whereby a transcription factor is functionally reconstituted via interaction of two proteins of interest, which in turn triggers the transcription of marker genes from the yeast genome.

For example, when using the GAL4 transcription factor, two expression plasmids are used and transformed into a yeast strain that carries a lacZ reporter gene regulated by GAL4 responsive elements. One plasmid contains the fusion gene required for the 'bait' protein to bind with the GAL4-binding domain. The other plasmid contains the fusion gene that codes for the 'prey' protein to fuse with the GAL4-activation domain. The production and activity of β-galactosidase, due to the expression of lacZ gene, indicates the interaction of the bait and prey proteins that results in the functional reconstitution of the GAL4 transcription factor [11].

Advantages: Y2H is a robust and user-friendly method to detect *in vivo* PPI at a comparatively lower cost, and with high-throughput. It has also low sample requirements as the proteins are produced and interact within the nucleus of the host cell itself.

Limitations: Conversely, Y2H is only suitable for studying interactions that occur in the nucleus. Additionally, post-translational modifications on the fusion proteins are specific to yeast and are not always applicable to mammalian hosts. The fusion proteins that are expressed at different locations or at different times can also come into contact and interact to give a false positive, while some 'bait' proteins can be toxic [12]. Furthermore, one important limitation may be caused by the autoactivation of genes or 'leaky' expression, which can lead to false positives.

3. Membrane yeast two hybrid

Membrane yeast two hybrid (MYTH) is a Y2H-modified method designed to study interactions of membrane proteins. In this method, a full length 'pseudo-ubiquitin' molecule is reconstituted when the 'bait' protein interacts with the 'prey' protein. The 'bait' protein is fused with the C-terminal part of yeast ubiquitin (Cub) which in turn is linked to an artificial transcription factor. The prey protein is fused to the N-terminal part of yeast ubiquitin (Nub) [13]. Interaction between the 'bait' protein and the 'prey' protein leads to the reassembly of a complete ubiquitin molecule. The transcription factor is then cleaved off by the activity of cytosolic deubiquitinating enzymes, thereby inducing reporter gene expression which can be analyzed to confirm the interaction [14].

Advantages: MYTH has proven to be an inexpensive, user-friendly method for providing high-throughput results in the study of membrane protein interactions without specialized equipment or infrastructure.

Limitations: The 'bait' and the 'prey' proteins interact only if they are expressed at the yeast plasma membrane along with other interacting proteins, hence the use of soluble proteins is not possible (the latter fail to reassemble the ubiquitin). Furthermore, post-translational modifications are specific to the host cell and are not the same in other hosts, which may affect the overall interaction pattern.

4. Protein arrays

This technique allows the study of multiple interactions simultaneously, with a high throughput. Hundreds to thousands of PPI are studied by immobilizing proteins on solid supports (nitrocellulose membranes, beads or microtiter plates) and subsequently subjected them to interaction with ligands, including potential proteins of interest [15]. *In situ* cell-free expression is also another option where genes of interest or cDNA are transcribed and translated *in vitro* onto the array [16]. The interaction is verified and analyzed by the use of fluorescent tagged probes added to the protein array.

Advantages: Protein arrays are fast and cost effective, and permit simultaneous study of multiple protein interactions. Furthermore, arrays are amenable to automation and demand low sample consumption to study multiple interactions.

Limitations: Transient protein interactions are undetectable, while immobilization of proteins on a solid support may influence their structure, thus affecting activity. Additionally, specificity of immobilized protein may not reflect *in vivo* conditions. There is also a loss of post-translational modifications if proteins are produced via recombination in *E. coli*, or different modifications than those in the mammalian hosts, when proteins are produced in yeast.

5. 2D gel electrophoresis

In order to analyze and quantify protein complexes formed via interaction between two or more proteins, 2D gel electrophoresis is combined with mass spectrometry. This method is an improvement on 1D gel electrophoresis, in that it allows the separation of protein complexes from a fractionated protein mixture after immunoprecipitation. In the first dimension separation is performed according to isoelectric point followed by a separation in the second dimension by molecular weight [17]. Individual proteins spots that constitute the protein-complex are then extracted from the gel and digested into peptides which are identified by mass spectrometry.

Differential gel electrophoresis is an adaptation of 2D electrophoresis, where a fluorescent dye of distinct wavelength covalently linked to the proteins is used to label each sample [19]. This increases the reproducibility and has a more dynamic range than traditional 2D PAGE assay. Relative quantitative assay can be carried out by using an internal standard for protein quantization.

Advantages: 2D gel electrophoresis is an inexpensive assay that can provide high resolution by using different pH ranges, and can effectively use crude extracts as samples. It can be complemented with mass spectrometry to study PPI and is automated for spot detection and alignment.

Limitations: It is a time-consuming technique with low throughput. Furthermore, it has a low reproducibility, and thereby a high degree of variability.

6. Atomic force microscopy

With the atomic force microscope (AFM), a probe attached to a cantilever scans a surface at a set distance or force. When both the surface and the cantilever are functionalized with the proteins of interest, the interaction between them can be measured in order to characterize their binding affinity under different force conditions [20].

Advantages: The method is useful for a relative comparison of interaction strengths of the partner proteins, with high resolution in the piconewton range [21]. AFM can be used for drug screening and kinetic studies.

Limitations: This technique has low throughput, and requires an expensive setup. Although providing high resolution images, these are small in size and may not reflect overall sample heterogeneity. Furthermore, protein adsorption may induce denaturation or may impose steric limitations on the protein movements.

7. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) is a quantitative technique that measures real time kinetics of PPI by determining the thermodynamic changes upon protein binding [7]. The equipment consists of a sample cell and a reference cell which are enclosed into an adiabatic jacket. The protein of interest is introduced in the sample cell, while the reference cell contains the buffer in which the sample is dissolved. The protein of interest is titrated against its partner, leading to uptake or release of heat, depending on the reaction. Sensitive thermopile/thermocouple circuits are used to detect thermal differences between the cells, which are analyzed to characterize the interaction. Enthalpy, entropy and changes in heat capacity of interaction can be derived along with the binding affinity of the proteins [22].

Advantages: ITC can be used to study affinity constants down to high picomolar range.

The equipment is highly thermo-sensitive as can detect heat changes as low as 0.1 mcal (0.4 mJ). ITC can be exploited to obtain PPI kinetic values with the reaction stoichiometry [23]. The proteins can be studied in their natural conformation in solution, as they are not bound on solid surface, which might limit their flexibility and activity.

Limitations: ITC requires highly purified proteins and may require high sample consumption, depending on the reaction under study. It is a time-consuming assay of low throughput, and PPI of very high (low picomolar) affinity may not be described accurately.

8. Backscattering interferometry and Bio-layer interferometry

Backscattering interferometry (BSI) is a quantitative method that measures the changes in the refractive index resulting from the interaction of two protein molecules, either in solution or adsorbed [24]. This is analyzed by studying the backscattered interference pattern generated by a laser light that illuminates the microfluidic channel containing the protein couple of interest. BSI is similar to ITC in the way that it facilitates analysis in solution [25], allowing measurements in the picomolar range for the quantification of binding events. Although both techniques detect refractive index changes, BSI is a bulk sensor that differs from Surface plasmon resonance (SPR), which has a sensor surface with a higher specificity.

In bio-layer interferometry (BLI), one surface is made of a layer of immobilized protein of interest on the biosensor tip. The second surface is an internal reference layer. The change in the number of partner proteins that interact with the protein bound to the biosensor tip leads to a shift in the interference pattern [26]. Real time measurement of this pattern is possible, thus enabling real time study of PPI. Because of the array format of BLI, kinetic rates and affinity constants of PPI can be measured in a shorter period, with specificity similar to SPR.

Advantages: BSI is a label free technique that is highly sensitive and therefore requires small sample volumes. BSI and BLI are also compatible with crude samples, without the need for surface attachment optimization. In addition, BLI has high throughput due to its array format.

Limitations: To proceed with either BSI or BLI, special infrastructure is required. As compared to other techniques BSI is a low-throughput method. Even though BLI is high-throughput, highly specific protein attachment is a prerequisite.

9. Circular dichroism

Circular dichroism (CD) method relies on changes in the spin angular momentum resulting from the differential absorption of left- and right-handed circularly polarized light. CD is exhibited by any optically active molecule, therefore proteins can exhibit CD due to their dextrorotary or levorotary characteristics [27]. Since the interaction of a protein of interest with another protein affects the protein conformation, the CD spectrum shifts and can be examined at various concentrations of the binding partner at different time points, thereby providing information about the binding constant and thermodynamics of interaction [28].

Advantages: CD is a technique that allows label-free detection with high specificity, while also providing information on the kinetics of the PPI. Proteins can be studied in their native form in solution as CD does not require protein immobilization on a surface.

Limitations: Although CD gives high specificity, it has low throughput. CD has high sample consumption and requires highly pure proteins. The analysis of PPI by CD spectroscopy is only suitable for completely dissolved proteins, and the method has low structural resolution and low sensitivity to structural changes, when compared to X-ray crystallography and NMR spectroscopy. Common aqueous buffer systems like phosphate, sulfate, carbonate and acetate are not compatible unless used in low millimolar concentration, as they often absorb in the range where structural features of PPI exhibit their typical CD spectra.

10. Surface plasmon resonance

Surface plasmon resonance (SPR) method is an affinity-based technique that uses refractive-index surface sensors, enabling real time detection and quantification of PPI. In SPR, one protein of interest is adsorbed and its partner protein, present in aqueous buffer solution, is titrated against [29]. As the proteins interact, the functionalized surface alters the angle at which polarized light is reflected. An inverse phenomenon is observed during dissociation of the protein couple, allowing for the determination of the kinetic constants.

Advantages: SPR permits label-free detection and real-time kinetic measurements. Purified protein sample is not mandatory for SPR analysis as it can be coupled to MS to identify the partner interaction and detection. SPR has very high sensitivity, especially for typical high affinity antigen-antibody complexes, and for drug discovery [30].

Limitations: SPR gives low throughput and is not suitable for detection of low-molecular weight proteins, as mass transport can affect kinetic analysis. Similar to other surface interaction techniques, one protein interaction partner is immobilized which might modify the protein tertiary and quaternary structure and potentially lead to a loss of function/activity.

11. Nuclear magnetic resonance

Nuclear magnetic resonance technique (NMR) is an established biophysical technique employed for the study of intermolecular interactions. The three dimensional structure of the protein-protein complex can be studied by heteronuclear single quantum coherence spectrum of isotopically labeled ^{13}C, ^{15}N, ^{2}H. By examining the chemical shifts in the resonance of amino acids, upon addition of the unlabeled partner protein, NMR can help identify and localize the binding sites [31]. NMR can also be used for studying transient interactions [32].

Another parameter analyzed is the nuclear Overhauser effect, used to measure the distance between two protons. NMR technique in 1D or 2D allows the analysis of slow, intermediate or fast PPI, by titrating one protein against its partner protein of interest [33].

Advantages: NMR provides high-resolution data compared to other available techniques like CD. NMR can also detect weak PPI and is highly sensitive (affinities in the low nanomolar range). NMR is compatible with liquid or crystallized protein and can be combined with functional assays.

Limitations: Incorporation of ^{13}C or ^{15}N in recombinant proteins is limited to specific media. NMR has limitations on the protein sizes and complexes that can be used, and can only characterize small and soluble proteins, with high sample consumption.

12. Microscale thermophoresis

Microscale thermophoresis (MST) can study PPI in near-native conditions using the thermophoretic effect, by allowing measurements of interactions in artificial buffer systems, body liquids or cell lysates [34]. Thermophoresis directs the movement of molecules in a microscopic temperature gradient. When a change in the conformation of a given protein due to interaction with a partner occurs, a corresponding change in its motion along the temperature gradient is enacted. These events can be detected by MST and the data can then be used to determine binding affinities, with low sample consumption [35].

Advantages: MST allows the measurement of binding affinities for a PPI. No purified protein is needed and measurements are possible under near-natural conditions.

Limitations: MST has low specificity. The method is highly sensitive to various changes in physical molecular properties like charge and size, which leads to high background noise.

13. Fluorescence polarization (Anisotropy spectroscopy)

This technique quantifies the changes in fluorescence polarization of a fluorophore attached to the protein of interest, while it undergoes rotational or translational movements during excitation. Unequal emission intensities are obtained along different axes of polarization that depend directly on the size of the fluorophore, thus revealing information on

specific PPI [36]. Parameters such as temperature or solution viscosity are varied to gauge the extent of fluorophore movement.

Advantages: Anisotropy spectroscopy allows PPI measurements in real time with a high dynamic range. High throughput can be achieved by using microfluidic system (picoliter volumes).

Limitations: A major limitation is the stability of the fluorophore in the excited state for longer periods. Being highly sensitive, the signal is influenced by the size and shape of a molecule. Solvent viscosity and maintenance of the homogeneity of solution must be considered in analyzing data, as they can directly affect the movement of a fluorophore [37].

14. Mass spectrometry

Mass spectrometry (MS) is a commonly used technique in conjunction with other PPI analysis techniques, in order to analyze the protein structure, including post-translational modifications, and for relative quantification of proteins in a sample. Three major processes occur within the mass spectrometer: (1) ionization (2) separation of ions based on mass to charge (m/z) ratio (3) detection of ions.

Partially purified proteins by electrophoresis or co-immunoprecipitation are enriched (especially in the case of low abundance proteins) and later digested using eg. trypsin. MS analyzes the mass/charge ratio of sample peptides and can detect protein modifications like phosphorylation, by finding the mass variations of the digested peptides [38]. Relative quantification is possible by comparing protein samples via label-free or isotope labeling. Label-free techniques include peak-intensity analysis with respect to the retention time and via spectral counting. Absolute quantification is only possible if the peak sizes of test protein complex sample are compared with a known internal reference protein sample [39].

Labeling techniques coupled with MS

14.1. L-Azidohomoalanine (AHA)

Advanced co-immunoprecipitation has been achieved by using L-AHA (methionine surrogate) to study newly synthesized proteins and their partners [40]. AHA is incorporated in proteins during translation instead of methionine, when cells are cultured in its absence. The non-toxic AHA does not hinder the function of the newly synthesized proteins nor changes their conformation. The AHA containing protein interacts with its partner proteins and the complex can be co-immunoprecipitated for further study. A chemo-selective reaction between the AHA-protein and an alkyne is used to detect the newly synthesized protein.

Advantages: This technique allows the monitorization of *in vivo* protein interactions via the use of selective and non-toxic labeling [41]. AHA is available commercially as an inexpensive marker. Proteins can be studied in their native conditions and the technique can be coupled with mass spectrometry for further analysis.

Limitations: Cells need to be starved of methionine and the technique is dependent on MS for further analysis of PPI.

14.2. Stable-isotope labelling by amino acids in cell culture (SILAC)

Heavy isotopes ($^{15}N/^{13}C$) are used to label amino acids that are incorporated into the protein of interest. Due to their presence, MS analysis can thus be used to detect the ensuing mass changes [42].

Advantages: This technique permits *in vivo*, non-radioactive labeling and has been proven to be a robust and sensitive method.

Limitations: SILAC must be used with mass spectrometry, therefore heavy isotopes containing media are required for incorporation in the proteins.

15. Förster resonance energy transfer

Förster resonance energy transfer (FRET) is an experimental method that studies the interaction between two proteins that are each conjugated with either an acceptor or a donor fluorophore [43]. When the excitation energy of the donor overlaps with the absorption energy of the acceptor, there is an energy transfer from the donor fluorophore to the acceptor fluorophore [44].

Advantages: Studying PPI *in vivo* at single-molecule resolution with real-time observation of protein complexes is possible. With dual wavelength detecting, standard microarray scanners are compatible with FRET detection, a method that is highly sensitive and able to achieve the cellular localization of the interaction of interest.

Limitations: FRET is still low throughput and only detects PPI when their distance is within a a range of 10 nm in live or fixed cells. Proteins of interest have to be conjugated with fluorophores for the emission-detection, whose use can alter function, location and conformation of proteins being studied. This method is not suitable for proteins expressed in low concentrations, and it can give potentially high background due to autofluorescence.

16. Bimolecular fluorescence complementation

Bimolecular fluorescence complementation (BiFC) is a modification of the protein assays like Y2H, whereby the intensity signal generated by a fluorescent protein is measured and analyzed. The fluorescent protein is reconstituted from two non-fluorescent protein fragments [45].

Advantages: A simple fluorometer or cell cytometer can be used to measure the fluorescence intensities. Fluorescence microscopy can be used to analyze the PPI and its localization *in vivo* [46]. The method has high sensitivity and permits detection of weak and transient interactions with low background noise.

Limitations: The stability of reconstituted fluorophore is generally low. Various non-specific interactions can give rise to false positive results, and this method cannot detect dynamic changes in the PPI during or post-interaction.

17. Fluorescence correlation spectroscopy

In fluorescence correlation spectroscopy (FCS), fluorescently labeled molecules are excited in a small detection volume ranging from picoliters to femtoliters. The intensities of excited molecules fluctuate as these diffuse in and out of the detection volume, and are measured to provide information regarding the movement patterns [47].

When a fluorescently labeled protein binds to other protein, its mobility is restricted, affecting its fluctuation rate. When detected by confocal microscopy, FCS allows characterization of PPI *in vivo* at single molecule sensitivity [48]. A modification to FCS which proves to be more accurate is fluorescence cross-correlation spectroscopy (FCCS) that labels both of the interaction partners [49].

Advantages: FCS is a highly specific method requiring very small sample volumes and low concentration of proteins in picomolar range. The method has single molecule sensitivity and allows study of PPI *in vivo*.

Limitations: Photo-bleaching can cause loss of signal. As with BiFC, the fluorophore has low stability.

18. Proximity ligation assay

With proximity ligation assays (PLA), upon interaction of the proteins of interest, oligonucleotide amplification is induced, resulting in production of detectable DNA molecules which can be analyzed via PCR or fluorescence microscopy [50]. When their binding sites are in sufficient proximity, an interaction between their single strand oligonucleotides occurs, which is helped by a third DNA oligonucleotide complementary to the ends of both strands [51]. The resulting polynucleotide DNA amplifies via the rolling circle model, incorporating fluorescent dyes simultaneously in the presence of DNA replisome and appropriate fluorescent substrates. Fluorescence microscopy can be used to analyze the PPI with single molecule sensitivity [52]. The PPI give rise to detectable DNA molecules containing the specific oligonucleotide sequences that can be further analyzed via PCR.

Advantages: PLA can detect ternary complexes, and requires small amount of samples. PLA can be used to study PPI in tissue sections with high sensitivity, and in their native state.

Limitations: No information can be obtained on the direct interaction of two proteins except that they co-localize. PLA requires high-

ly specific antibodies and there are antibody background problems when fluorescent microscopy is performed. Furthermore, PLA has no multiplexing capacity.

19. Fluorescence microscopy techniques

Fluorescence microscopy techniques (FM) can reveal the location, movement, and interactions of proteins in fixed and living cells. This method uses a fluorescent tag, eg. an antibody that labels the protein of interest and allows its study. However, the fluorescent tag can affect the subcellular location and the conformation of the protein.

Advantages: This method allows the real-time study of proteins, their location and interactions with high specificity and sensitivity. It can also detect transient PPI. Affinity tagging can be coupled with this method to make it high throughput.

Limitations: FM is strictly dependent on the antibody/fluorophore specificity.

19.1 Fluorescence recovery after photobleaching

With fluorescence recovery after photobleaching (FRAP), the region of interest is photo-bleached and then the motility of the fluorescent protein of interest is studied [53]. If the protein of interest interacts with a partner, its motility will be affected. This method allows *in vivo* detection of the intracellular motility of targeted protein [54]. However, specific instrumentation is required and the temperature has to be well regulated for the photo-bleaching. Moreover, this method does not allow one to study specificity of PPI.

19.2 Fluorescence lifetime imaging microscopy

Fluorescence lifetime imaging microscopy technique (FLIM) is used to study dynamic PPI, by monitoring the fluorescence lifetime of a probe of interest. A change in the probe fluorescence suggests an interaction with a partner protein, thereby allowing real time monitoring [55], especially in combination with FRET, in order to obtain more information. However FILM is not affected by signal contamination and can resolve the increase in FRET population [56]. However, as for all fluorescence microscopy techniques, it requires expensive equipments and is not reliable for fixed cells.

19.3 Bioluminescence resonance energy transfer

Bioluminescence resonance energy transfer (BRET) is used to detect PPI based on the luciferase activity, when a partner protein is conjugated with the fluorescent protein of interest. This method allows real time detection of PPI in both cell culture and deep tissue and reduces the number of false negative results [57]. However, it faces limitation due to light attenuation during tissues analysis and requires specific equipment [58]. Furthermore, the weak PPI that take place in deep tissues are not experimentally available.

20. X-ray crystallography and single particle electron microscopy

Similar to NMR, X-ray crystallography and single particle electron microscopy (EM) enable resolving structural details of proteins, by providing an atomic description of protein binding interfaces [59]. X-ray crystallography is used to analyze crystallized proteins in their functional states [8], while EM allows the study of proteins that are difficult to crystallize in specific functional states, at a comparable resolution.

Advantages: This method provides high resolution at an atomic level and can detect conformational changes.

Limitations: Low molecular weight proteins and complexes are not resolvable. Proteins or the protein complex of interest need to be crystallized, which can affect native conformation, and weak protein–protein interactions can be lost.

II. *Ex vivo* techniques for the study of PPI

1. In-silico pathway guides

These databases provide information on various biological pathway related resources and molecular interaction related information. **Reactome** [60] is a free, open-source, curated and peer reviewed pathway database provid-

ing bioinformatics tools for the visualization, interpretation and analysis of pathway knowledge along with PPI that make up the pathways. **Pathway studio** [61] is a pathway database based on the text mining technology that processes vast collection of published data, automatically extracting biological terms, concepts, and relationships on the PPI.

2. Experimental databases

Various databases are available online to allow *in silico* analysis of PPI that were studied either computationally using available structural information (String), algorithms (pre-PPI) or genomic data, known PPI networks or sequence information [62].

These may include: DIP (database of interacting proteins) [63], Biomolecular Interaction Network Database (BIND) [64], Biological General Repository for Interaction Datasets (BioGRID) [65], Munich Information centre for Protein Sequences (MIPS) [66].

These databases provide high quality data on experimentally determined interactions between proteins, protein complexes and pathways. They also give data on relationships between protein interactions and domain architecture along with various tools and resources. However, these databases are limited to two-protein interactions and do not reveal if the interaction is direct, nor do they distinguish between various protein isoforms.

3. Prediction databases: Search tool for the retrieval of interacting genes/proteins (STRING)

STRING provides data on known and predicted PPI including information on the physical (direct) and functional (indirect) associations of proteins [67]. More than 1.000 completely sequenced organisms are available for reference.

Data on protein of interest is available, however transient interactions are difficult to predict, whereas the pre-PPI database can provide **s**tructure information of protein–protein interactions along with predicted and experimentally determined PPI.

CONCLUSIONS

With only around 10% of all human PPI identified thus far [68], experimental validation of potential protein-protein interactions is essential for the biomedical field. As each experimental method comes with unique advantages and limitations, a set of techniques is necessary to provide for a full description of PPI. However, it is important to state that when used in combination, the failure of any particular experimental technique to show an interaction does not dismiss the others′ positive results [69].

With the fast advances in big data, mining this vast array of information remains challenging and demands a complementation of *in silico* studies with experimental data, in order to better understand the system under study.

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REFERENCES

1. Quax TE, Claassens NJ, Söll D, van der Oost J. Codon Bias as a Means to Fine-Tune Gene Expression. Mol Cell. 2015;59(2):149-61.
2. Csizmok V, Follis AV, Kriwacki RW, Forman-Kay JD. Dynamic protein interaction networks and new structural paradigms in signaling. Chem Rev. 2016;116(11):6424–6462.
3. Nooren IMA, Thornton JM. Diversity of protein-protein interactions. EMBO J. 2003;22 (14):3486–3492.
4. 4. Garma L, Mukherjee S, Mitra P, Zhang Y. How many protein-protein interactions types exist in nature? PloS One. 2012;7(6):e38913.
5. Ideker T, Sharan R. Protein networks in disease. Genome Res. 2008;4:644-52.
6. Perkins JR, Diboun I, Dessailly BH, Lees JG, Orengo C. Transient protein-protein interactions: structural, functional, and network properties. Struct Lond Engl. 1993. 2010;18(10):1233–1243.
7. Keskin O, Gursoy A, Ma B, Nussinov R. Principles of protein-protein interactions: what are the preferred ways for proteins to interact? Chem Rev. 2008;4:1225-44.
8. Gandhi TK, Zhong J, Mathivanan S, Karthick L, Chandrika KN, Mohan SS, et al. Analysis of the human protein interactome and comparison with yeast, worm and fly interaction datasets. Nat Genet. 2006;38:285–293.

9. Hage DS, Matsuda R. Affinity chromatography: a historical perspective. Methods Mol Biol. 2015;1286:1–19.
10. Gerace E, Moazed, D. Affinity purification of protein complexes using TAP tags. Methods Enzymol. 2015;559:37–52.
11. Mehla J, Caufield JH, Uetz P. The yeast two-hybrid system: a tool for mapping protein-protein interactions. Cold Spring Harb Protoc. 2015;5:425-30.
12. Caufield JH, Sakhawalkar N, Uetz P. A comparison and optimization of yeast two-hybrid systems. Methods San Diego Calif. 2012;58(4):317–324.
13. Snider J, Kittanakom S, Curak J, Stagljar I. Split-Ubiquitin based membrane yeast two-hybrid (MYTH) system: A Powerful Tool For Identifying Protein-Protein Interactions. J Vis Exp JoVE. 2010;(36).
14. Snider J, Stagljar I. Membrane Yeast Two-Hybrid (MYTH) Mapping of full-length protein interactions. Cold Spring Harb. Protoc. 2016;(1) pdb.top077560.
15. Berrade L, Garcia AE, Camarero JA. Protein microarrays: novel developments and applications. Pharm Res. 2011;28(7):1480–1499.
16. Schinn SM, Broadbent A, Bradley WT, Bundy BC. Protein synthesis directly from PCR: progress and applications of cell-free protein synthesis with linear DNA. N Biotechnol 2016;33(4):480-7.
17. Westermeier R. Looking at proteins from two dimensions: a review on five decades of 2D electrophoresis. Arch. Physiol. Biochem. 2014;120(5):168–172.
18. Grandjean M, Dieu M, Raes M, Feron O. A new method combining sequential immunoaffinity depletion and differential in gel electrophoresis to identify autoantibodies as cancer biomarkers. J Immunol Methods, 2013;396(1–2):23–32.
19. Ohlendieck K. Comparative DIGE proteomics. Methods Mol Biol Biochim Biophys Acta Proteins Proteom. 2019;1867(1):2-8.
20. Whited AM, Park PS-H. Atomic force microscopy: a multifaceted tool to study membrane proteins and their interactions with ligands. Biochim Biophys Acta 2014;1838(1 Pt A):56–68.
21. Fahs A, Louarn G. Plant protein interactions studied using AFM force spectroscopy: nanomechanical and adhesion properties. Phys Chem Chem Phys. PCCP. 2013;15(27):11339–11348.
22. Krell T, Lacal J, García-Fontana C, Silva-Jiménez H, Rico-Jiménez M, Lugo AC, et al. Characterization of molecular interactions using isothermal titration calorimetry. Methods Mol Biol. 2014;1149:193–203.
23. Rajarathnam K, Rösgen J. Isothermal titration calorimetry of membrane proteins - progress and challenges. Biochim Biophys Acta. 2014;1838(1 Pt A):69–77.
24. Baksh MM, Kussrow AK, Mileni M, Finn MG, Bornhop DJ. Label-free quantification of membrane-ligand interactions using backscattering interferometry. Nat Biotechnol 2011;29(4):357–360.
25. Bornhop DJ, Latham JC, Kussrow A, Markov DA, Jones RD, Sørensen HS. Free-solution, label-free molecular interactions studied by back-scattering interferometry. Science. 2007;317(5845):1732–1736.
26. Shah NB, Duncan TM. Bio-layer interferometry for measuring kinetics of protein-protein interactions and allosteric ligand effects. J Vis Exp JoVE. 2014;(84):e51383.
27. Micsonai A, Wien F, Kernya L, Lee Y-H, Goto Y, Réfrégiers M, et al. Accurate secondary structure prediction and fold recognition for circular dichroism spectroscopy. Proc Natl Acad Sci USA. 2015;112(24):E3095-3103.
28. Greenfield NJ. Circular dichroism (CD) analyses of protein-protein interactions. Methods Mol Biol. 2015;1278:239–265.
29. Nikolovska-Coleska Z. Studying protein-protein interactions using surface plasmon resonance. Methods Mol Biol. 2015;1278:109–138.
30. Patching SG. Surface plasmon resonance spectroscopy for characterisation of membrane protein-ligand interactions and its potential for drug discovery. Biochim Biophys Acta. 2014;1838(1 Pt A):43–55.
31. Cala O, Guillière F, Krimm I. NMR-based analysis of protein-ligand interactions. Anal. Bioanal Chem. 2014;406(4):943–956.
32. Liu Z, Gong Z, Dong X, Tang C. Transient protein-protein interactions visualized by solution NMR. Biochim Biophys Acta. 2016;1864(1):115–122.
33. Aguirre C, Cala O, Krimm I. Overview of probing protein-ligand interactions using NMR. Curr Protoc Protein Sci. 2015;81:17.18.1-24.
34. Mao Y, Yu L, Yang R, Qu L, Harrington P de B. A novel method for the study of molecular interaction by using microscale thermophoresis. Talanta. 2015;132:894–901.
35. Asmari M, Ratih R, Alhazmi HA, El Deeb S. Thermophoresis for characterizing biomolecular interaction. Methods. 2018;146:107-119.
36. Du Y. Fluorescence polarization assay to quantify protein-protein interactions in an HTS

format. Methods Mol Biol. 2015;1278:529–544.
37. Raines RT. Fluorescence polarization assay to quantify protein-protein interactions: an update. Methods Mol Biol. 2015;1278:323–327.
38. Mehmood S, Allison TM, Robinson CV. Mass spectrometry of protein complexes: from origins to applications. Annu Rev Phys Chem. 2015;66:453–474.
39. Syafrizayanti, Betzen C, Hoheisel J.D, Kastelic D. Methods for analyzing and quantifying protein–protein interaction. Expert Rev Proteomics. 2014;11(1):107–120.
40. Schiapparelli LM, McClatchy DB, Liu H-H, Sharma P, Yates JR, Cline HT. Direct detection of biotinylated proteins by mass spectrometry. J. Proteome Res 2014;13(9):3966–3978.
41. Hayes S, Malacrida B, Kiely M, Kiely PA. Studying protein-protein interactions: progress, pitfalls and solutions. Biochem Soc Trans. 2016;44(4):994–1004.
42. Emmott E, Goodfellow I. Identification of protein interaction partners in mammalian cells using SILAC-immunoprecipitation quantitative proteomics. J Vis Exp JoVE 2014; (89).
43. Margineanu A, Chan JJ, Kelly DJ, Warren SC, Flatters D, Kumar S, et al. Screening for protein-protein interactions using Förster resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM). Sci Rep 2016;6:28186.
44. Benz C, Retzbach H, Nagl S, Belder D. Protein-protein interaction analysis in single microfluidic droplets using FRET and fluorescence lifetime detection. Lab Chip. 2013;13(14):2808–2814.
45. Pham CD. Detection of protein-protein interaction using bimolecular fluorescence complementation assay. Methods Mol Biol. 2015;1278:483–495.
46. Lee L-Y, Gelvin SB. Bimolecular fluorescence complementation for imaging protein interactions in plant hosts of microbial pathogens. Methods Mol Biol. 2014;1197:185–208.
47. Bacia K, Haustein E, Schwille P. Fluorescence correlation spectroscopy: principles and applications. Cold Spring Harb Protoc. 2014;(7):709–725.
48. Hink MA. Fluorescence correlation spectroscopy. Methods Mol Biol. 2015;1251135–150.
49. Padilla-Parra S, Audugé N, Coppey-Moisan M, Tramier M. Quantitative study of protein-protein interactions in live cell by dual-color fluorescence correlation spectroscopy. Methods Mol Biol. 2014;1076:683–698.
50. Chen T-C, Lin K-T, Chen C-H, Lee S-A, Lee P-Y, Liu Y-W, et al. Using an in situ proximity ligation assay to systematically profile endogenous protein-protein interactions in a pathway network. J. Proteome Res. 2014;13(12):5339–5346.
51. Bellucci A, Fiorentini C, Zaltieri M, Missale C, Spano P. The "in situ" proximity ligation assay to probe protein-protein interactions in intact tissues. Methods Mol Biol. 2014;1174:397–405.
52. Ke R, Nong RY, Fredriksson S, Landegren U, Nilsson M. Improving precision of proximity ligation assay by amplified single molecule detection. PloS One. 2013;8(7):e69813.
53. Fritzsche M, Charras G. Dissecting protein reaction dynamics in living cells by fluorescence recovery after photobleaching. Nat Protoc. 2015;10(5):660–680.
54. Bolognesi A, Sliwa-Gonzalez A, Prasad R, Barral Y. Fluorescence Recovery After Photo-Bleaching (FRAP) and Fluorescence Loss in Photo-Bleaching (FLIP) Experiments to study protein dynamics during budding yeast cell division. methods Mol Biol. 2016;1369:25–44.
55. Sun Y, Periasamy A. Localizing protein-protein interactions in living cells using fluorescence lifetime imaging microscopy. Methods Mol Biol. 2015;1251:83–107.
56. Osterlund EJ, Liu Q, Andrews DW. The use of FLIM-FRET for the detection of mitochondria-associated protein interactions. Methods Mol Biol. 2015;1264:395–419.
57. Dimri S, Basu S, De A. Use of BRET to Study protein-protein interactions *in vitro* and *in vivo*. Methods Mol Biol. 2016;1443:57–78.
58. Deriziotis P, Graham SA, Estruch SB, Fisher SE. Investigating protein-protein interactions in live cells using bioluminescence resonance energy transfer. J Vis Exp JoVE. 2014;(87).
59. Miyaguchi K. Direct imaging electron microscopy (EM) methods in modern structural biology: overview and comparison with X-ray crystallography and single-particle cryo-EM reconstruction in the studies of large macromolecules. Biol Cell. 2014;106(10):323–345.
60. Croft D, Mundo AF, Haw R, Milacic M, Weiser J, Wu G, et al. The Reactome pathway knowledgebase. Nucleic Acids Res. 2014;42:D472-477.
61. Yuryev A, Kotelnikova E, Daraselia N. Ariadne's chem effect and pathway studio knowledge base. Expert Opin. Drug Discov. 2009;4(12):1307–1318.
62. Miryala SK, Anbarasu A, Ramaiah S. Discerning molecular interactions: A comprehensive review on biomolecular interaction databases and network analysis tools. Gene. 2018;642:84–94.

63. Salwinski L, Miller CS, Smith AJ, Pettit FK, Bowie JU, Eisenberg D. The database of interacting proteins: 2004 update. Nucleic Acids Res. 2004;32:D449-451.
64. Willis RC, Hogue CWV. Searching, viewing, and visualizing data in the Biomolecular Interaction Network Database (BIND). Curr. Protoc. Bioinforma. 2006;Chapter 8,Unit 8.9.
65. Islamaj Dogan R, Kim S, Chatr-Aryamontri A, Chang CS, Oughtred R, Rust J, et al. The BioC-BioGRID corpus: full text articles annotated for curation of protein-protein and genetic interactions. Database J Biol. Databases Curation, 2017.
66. Mewes HW, Ruepp A, Theis F, Rattei T, Walter M, Frishman D, et al. MIPS: curated databases and comprehensive secondary data resources in 2010. Nucleic Acids Res. 2011;39 (Database issue):D220-224.
67. Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, et al. STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic Acids Res. 2015; 43 (Database issue): D447-452.
68. Schoenrock A, Samanfar B, Pitre S, Hooshyar M, Jin K, Phillips CA, et al. Efficient prediction of human protein-protein interactions at a global scale. BMC Bioinformatics. 2014;15(1).
69. Xing S, Wallmeroth N, Berendzen KW, Grefen C. Techniques for the analysis of protein-protein interactions *in vivo*. Plant Physiology. 2016;171(2):727–758.