EXPERT REVIEWS

Methods for analyzing and quantifying protein–protein interaction

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Syafrizayanti[1,2], Christian Betzen[1,3], Jörg D Hoheisel[1] and Damjana Kastelic*[1]

[1]*Division of Functional Genome Analysis, Deutsches Krebsforschungszentrum (DKFZ), Im Neuenheimer Feld 580, Heidelberg, Germany*
[2]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Andalas University, Kampus Limau Manis, Padang, Indonesia*
[3]*Division of Nephrology, Department of Pediatrics, University Hospital, Im Neuenheimer Feld 430, Heidelberg, Germany*
**Author for correspondence: d.kastelic@dkfz.de*

Genome sequencing has led to the identification of many proteins, which had not been recognized before. In consequence, the basic set of human proteins is generally known. Far less information, however, exists about protein–protein interactions, which are required and responsible for cellular activities and their control. Many protein isoforms that result from mutations, splice-variations and post-translational modifications also come into play. Until recently, interactions of only few protein partners could be analyzed in a single experiment. However, this does not meet the challenge of investigating the highly complex interaction patterns in cellular systems. It is made even more demanding by the need to determine the intensity of interactions quantitatively in order to properly understand protein interplay. Currently available techniques vary with respect to accuracy, reliability, reproducibility and throughput and their performances range from a mere qualitative demonstration of binding to a quantitative characterization of affinities. In this article, an overview is given of the methodologies available for analysis of protein–protein interactions.

Keywords: assay formats • detection • interactions • proteomics • quantification

Although sequencing of 'the first human genome' was completed more than a decade ago, our understanding of the enormous number of functions arising from the three billion base pairs is far from complete [1]. The number of coding sequences was found to be surprisingly small compared with the apparent complexity of biological functions in a cell [2]. This implicates that other processes are important for generating the biological complexity, which translates into functional flexibility. Transcriptional control mechanisms, for example, regulate: gene expression levels, the generation of splice variants, the production of inhibitory RNA molecules and the expression and processing of other noncoding RNAs, which are, at least in part, responsible for particular functions, such as the recruitment of chromatin modification complexes to genomic loci. Transcript variants have also immediate consequences at the protein level, with splice variations encoding functionally distinct protein isoforms [3–5], for instance, or by differently affecting protein functions by interaction.

While all this is widening the functional landscape substantially, the large number of potential protein–protein interactions (PPIs) and their regulation in time and space is probably adding even more to the functional flexibility of cellular systems. On the basis of data from yeast two-hybrid (Y2H) experiments in combination with mammalian reverse two-hybrid technology and Bayesian modeling, the number of binary interactions between human proteins was estimated to number around 130,000 [6]. This may well be an underestimation, however. For a single pathway alone – MAPK pathway – 2269 protein interactions were proposed on the basis of again Y2H and small interference RNA analyses [7]. Moreover, a modeling approach proposed about 4000 types of protein complexes and concluded that the currently known protein structures only fit to 42% of the predicted protein complexes [8].

Protein interactions can be transient or of a more stable nature. Transient interactions are far less conserved mechanistically. They are the dominating form of binding events and represented in many protein families. They are basically involved in all kinds of processes, from the recruitment and assembly of transcription

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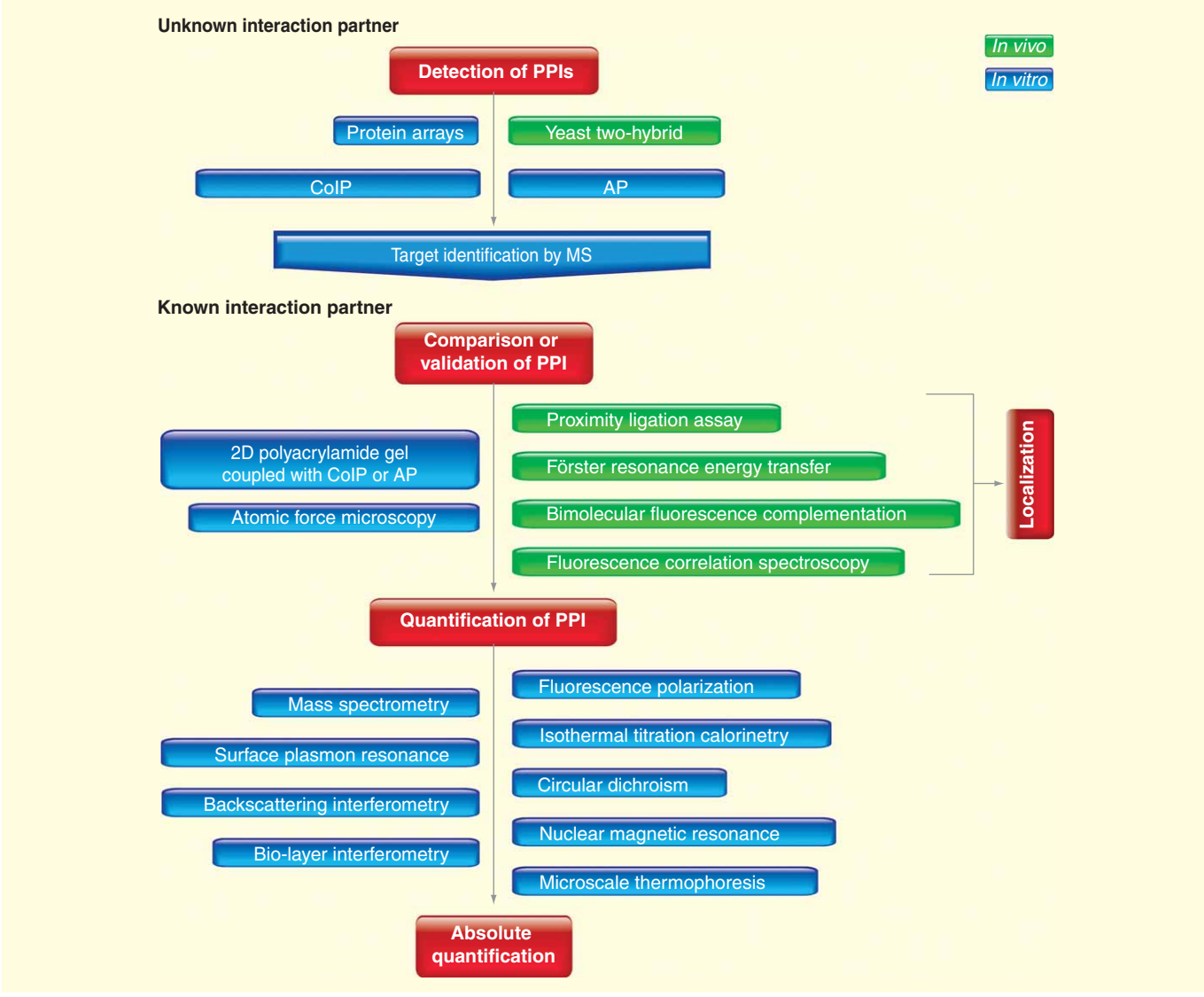

Figure 1. Application areas of the techniques for an analysis of protein–protein interactions.
AP: Affinity purification; CoIP: Co-immunoprecipitation; MS: Mass spectrometry; PPI: Protein–protein interaction.

complexes, via the transport of proteins across membranes, to the breakdown and reformation of subcellular structures like the spindle apparatus and the nuclear pore complex during cell division [9]. Some proteins constitute an integral part of a structure – such as in hemoglobin, a ribosome or proteasome – for others, the interaction is of a nonobligate nature, which means that each component of a complex does also exist and show activity on its own [10]. Often, a distinction between the states is rather fuzzy; instead, a continuum exists that depends on various parameters, such as pH, protein concentration or localization.

The methods applied for the analysis of PPIs have rather diverse objectives (Figure 1) and formats. A mere identification of binding events is still the most frequent purpose for analyses that aim at defining networks of interacting proteins and thus an initial description of functional pathways. However, the processes can also reveal the influence that a partner may have on the structure and thus function of the other one, including the alteration of kinetic properties, the formation of a new binding site, changes in the substrate specificity of an enzyme or the inactivation of a protein [11]. X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy [12] allow a very detailed structural investigation of protein interactions at an atomic resolution, providing a precise view of the interaction surface and yield information about amino acid contributions, the presence of salt bridges, hydrogen bonds and water molecules, which are important components of an interaction. At the other end of the scale are holistic 'interactome' approaches, which aim at identifying all biologically relevant interactions between proteins toward a fundamental understanding of systemic processes. Mapping the human interactome in healthy people and patients has already contributed substantially to the identification of potential drug targets [13–16].

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For a higher level of understanding the recognition events of PPI, the description of the binding thermodynamics is required that regulate association [17]. The strength of an interaction, once formed, is defined by the dissociation constant (K_d) or the affinity constant (K_a), which is the inverted value of K_d and commonly used for the description of antibody–antigen interactions [10]. Several proteomics methods provide such thermodynamics data in addition to kinetic data and stoichiometry information [18–20]. They are essential for a true modeling of proteome activity since adding a quantitative component.

As outlined above, an analysis of protein function is much more complex than the investigation of the biology of nucleic acids, and not only because the structures involved are much more diverse. Therefore, different types of assays are needed in order to grasp the various aspects of protein interaction and activity (Figure 2). An identification of a protein's conformations and the characterization of its interactions with other proteins are instrumental for a comprehensive description of its biological functions in the context of cellular activity and regulation [9]. Since most drugs act at the level of proteins, such knowledge is also important for the ability to affect diseases in a controlled and specific manner.

Below, we describe methods that are applied to identify PPIs and procedures for the quantitative elucidation of interaction characteristics. For the purpose of this review, we focus on PPIs defined by a physical interaction of two proteins in the absence of additional major mediators. This definition excludes a large number of PPIs that take place in an *in vivo* setting. Among these are interactions that require additional protein or nonprotein-binding partners for complex formation, as well as protein pairs that are linked functionally but not through a direct physical interaction, like proteins of the mitochondrial respiratory chain, for example. Also, in a partly biased and imprecise classification process, we subdivide the methods into procedures that define interactions at a qualitative level, permit relative quantification or allow for absolute quantification (Table 1). Given the current importance of mass spectrometry (MS), a separate paragraph provides an overview about MS-based procedures.

Qualitative description of PPIs

Affinity purification & co-immunoprecipitation

A simple but effective setup for the detection of protein interactions is affinity purification (AP); (Figure 2). A 'bait' protein is coupled covalently to a support matrix or tethered to it in a noncovalent but highly affine manner, for example, by using appropriate antibodies attached to the surface or by means of the biotin–streptavid in pair. After passing a protein extract through the matrix, only proteins that interact with the bait protein are retained and subsequently eluted at high salt condition or the addition of an appropriate competitor or detergent. The experimental setup allows high bait concentrations and may capture low-affinity interactions. One needs to keep in mind, however, that such interactions may not exist *in vivo* and were formed *in vitro* because of the experimental conditions only. This type of affinity assay was first published about 40 years ago to detect proteins that interact with *Escherichia coli* RNA polymerase. Later, this method was usually combined with MS for the identification of the binding partners [21,22]. The same principle can also be applied for *in vivo* studies. A recombinant bait protein, which is fused to a tag molecule, is expressed in a host cell. After cell disruption, the bait protein is purified by taking advantage of the tag. Simultaneously, to the bait protein, the proteins that are bound to it are co-isolated and can be characterized. A similar principle is used for co-immunoprecipitation. A specific antigen–antibody complex is added to a cell lysate. After precipitation of the antibody, any protein that formed a complex with the antigen is eluted and identifiable by MS [23].

While the above approaches are well suited for screening, the results should be assessed independently in order to prove that the identified PPIs take place in a cell and not as a consequence of experimental processes, such as cell lysis. Actin and DNase I, for example, react with each other at high specificity although they never encounter each other in a cell [24]. For improved performance of interaction analyses, procedural modifications are applied that involve attaching or cleaving chemical groups to alter the solubility or other properties of the original molecules. In particular, cross-linking is being used regularly, chemically joining two or more molecules by a covalent bond. Thereby, weak or transient PPIs can be detected that may otherwise evade detection [18].

Conclusions

- Strengths: low cost; low sample consumption; no major infrastructure is needed; weak or transient PPIs can be detected by *in vitro* AP; label-free approach.
- Weaknesses: high background; low sensitivity; a large quantity of bait protein is needed to detect low-affinity interactions.
- Limitations: hydrophobicity of proteins normally situated in different cellular compartments.

Yeast two-hybrid

The Y2H (Figure 2) is a sensitive *in vivo* genetic detection method for PPIs based on the reconstitution of a functional transcription factor that triggers transcription of marker genes in the yeast nucleus [25]. Y2H was originally introduced in the mid-1980s using the yeast GAL4 transcription factor. For an analysis, two expression plasmids are used and transformed into a yeast strain, which carries a *lacZ* reporter gene under the control of GAL4 responsive elements. From one plasmid, a fusion gene with the GAL4-binding domain is generated, named the *bait*; in the other plasmid, a protein is fused to the GAL4-activating domain, referred to as *prey*. Interaction between the bait and prey proteins can be detected via the activity of β-galactosidase, the product of the *lacZ* gene. Using libraries of both plasmid types permit the detection of every possible interaction. Other Y2H systems have been developed on the basis of yeast strains that utilize other

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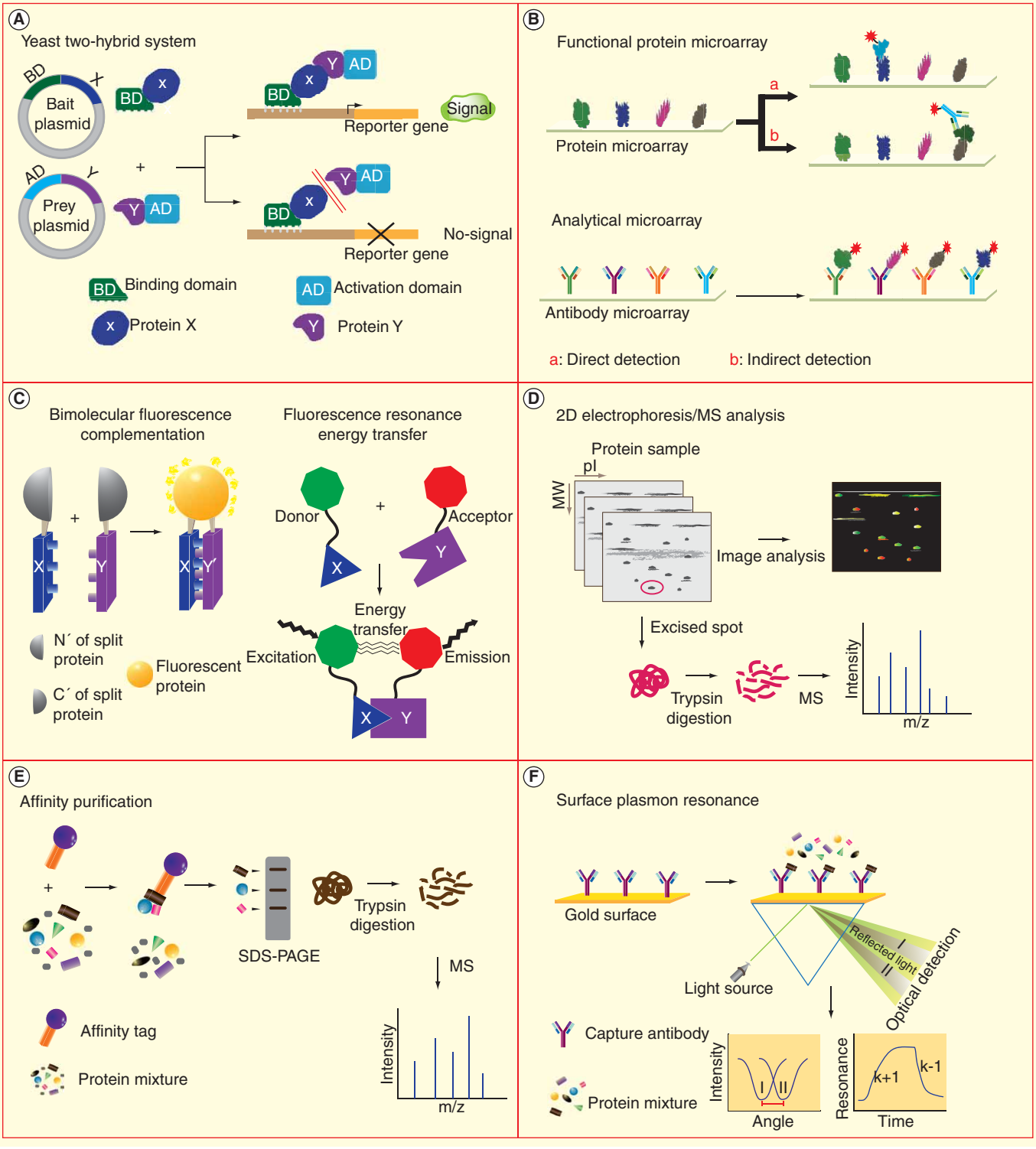

Figure 2. Schematic presentation of the processes of some of the methods discussed in the text.
MS: Mass spectrometry.

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Table 1. Comparison of protein–protein interaction methods.

Methods	Throughput	Specificity	Sensitivity	Sample consumption	Effort	Cost for infrastructure
Quantitative description of PPI						
Affinity purification	•	•	•	•	•	$
Co-immunoprecipitation	•	•	•	•	•	$
Yeast two hybrid	••	Very low	••	•	•••	$
Protein arrays	•••	•	••	•	•••	$$$
Detecting PPI intracellularly						
Förster resonance energy transfer	•	•••	••	N/A[†]	•••	$
Bimolecular fluorescence complementation	•	••	•••	N/A[†]	•••	$
Fluorescence correlation spectroscopy	•	••	•	N/A[†]	••	$
Proximity ligation	•	•••	•••	•	••	$
Relative quantification of PPI						
Two-dimensional polyacrylamide gel electrophoresis	•	•	•••	••	•••	$
Atomic force microscopy	•	N/A	•••	•	•••	$$$
Label free, absolute quantification of PPI						
Isothermal titration calorimetry	•	•/•••[‡]	•••	•••	•••	$$$
Backscattering interferometry	•	••	•••	•	•	$
Bio-layer interferometry	••	••	••	•	•	$
Circular dichroism	•	•••	••	••	•••	$$$
Surface plasmon resonance	•	•••	••	••	••	$$$
Nuclear magnetic resonance	•	•••	•	•••	•••	$$$$$
Microscale thermophoresis	•	•	•••	•	••	$$
Fluorescence polarization	••	•••	••	••	••	$$$
Mass spectrometry						
Mass spectrometry	•••	N/A[§]	•••	•	•••	$$$$$

•••: High; ••: Medium; •: Low.
[†]Proteins expressed *in vivo*.
[‡]Highly purified proteins required.
[§]Depends strictly on specificity of input method.
PPI: Protein–protein interaction.

reporter genes such as *HIS3, URA3, LEU2, ADE2, LYS2, gusA, GFP* and *MELI*.

Drawbacks of the Y2H system are – beside others – that the interaction takes place in the nucleus, the use of fusion proteins and the fact that proteins may come into contact that are naturally expressed at different locations or times. Several derivative systems have been developed to overcome the problems including son of sevenless recruitment, split-ubiquitin and dual-bait systems [26]. Also, new Y2H systems were established to study membrane PPIs [27]. Unfortunately, all Y2H systems are prone to yield false positives and false negatives. Estimates about the rate of false positives are as high as 70% [28].

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Nevertheless, Y2H in combination with MS has efficiently unraveled cellular networks of protein interactions at a subgenome or full genome level. The high-throughput potential of Y2H also provides a means for obtaining functional information of the interactome [29].

Conclusions
- Strengths: high throughput; low sample consumption.
- Weaknesses: many false positives and negatives; some infrastructure needs.
- Limitations: only suitable for studying PPIs that occur in nucleus; post-translational modifications are specific for yeast.

Protein arrays

The emergence of protein arrays (Figure 2) followed in the footsteps of DNA arrays. As an array-based technique, they offer the advantage of high throughput. Many interactions can be studied simultaneously; also the consumption of material is comparatively small. Functional protein arrays can contain hundreds to thousands of immobilized proteins on the solid support and are utilized for the identification of interactions with ligands like nucleic acids, proteins and peptides [30]. The latest development of protein array production and applications has been comprehensively reviewed elsewhere [31]. In a conventional approach, the proteins are produced in a cell-based system and transferred to the array after purification. This requires many handling steps and represents a major bottleneck. Alternatively, cell-free *in situ* expression is rapidly gaining popularity [32–34]. PCR products with all necessary regulative elements for transcription and translation are placed onto the array surface and only subsequently transcribed and translated *in vitro* directly on the surface [33,34]. Any cDNA library can thus be translated into a protein array [35]. Recent developments of prokaryotic and eukaryotic cell-free expression systems enable the synthesis of full-length proteins with high yields and a high percentage of functional molecules [36]. Irrespective of the production process, immobilization on a solid support will influence the structure of many molecules and thus affect protein activity. The simultaneously ongoing development of cell-like compartments holds the promise that more and more biologically active proteins could be generated in a cell-free manner. This would also permit a simpler creation of molecules with internal modifications, such as the addition of non-natural amino acids or processes for permutation experiments, which may support developments toward personalized medicine [37,38]. The most common detection mode when studying PPIs on protein arrays is fluorescence labeling [30,39]. For antibody microarrays, attomolar sensitivities have been reported [40] and even the detection of individual binding events is well in reach [41]. Alternatively, there are also label-free detection methods. For example, a high-density magnetoresistive sensor array was developed to quantify the kinetics of antibody–antigen binding at zeptomoles sensitivities [42].

Conclusions
- Strengths: multiplexing capacity; high throughput; low sample consumption.
- Weaknesses: possible loss of protein activity; infrastructure needed (spotter, scanner).
- Limitations: specificity of immobilized protein may not reflect *in vivo* situation; lack of post-translational modifications if recombinant proteins from *E. coli* or *in vitro* synthesized proteins are immobilized.

Detecting PPI intracellularly

In order to detect PPI in living cells and provide colocalization information, microscopy techniques such as Förster or fluorescence resonance energy transfer (FRET), bimolecular fluorescence complementation (BiFC) and fluorescence correlation spectroscopy (FCS) [43–45] are used for a direct observation of where and when proteins interact. The methods rely on the spectral emissions of fluorescent or chemiluminescent tags that are either fused with or conjugated to the proteins. Alternatives are proximity-induced ligation [46] or – similarly to the detection in Y2H experiments – a protein complementation assay [47].

Förster resonance energy transfer

More than six decades ago, FRET was introduced as a mechanism of electrodynamics energy transfer between donor and acceptor fluorophores, which are conjugated to the studied proteins (Figure 2). An energy transfer occurs when the excitation energy of the donor overlaps with the absorption energy of the acceptor [45]. The efficiency of the energy transfer is strongly distance dependent and thus only detectable at very close proximity of the two molecules in the range of a few nanometers. FRET has been used to monitor directly PPI in living cell [44]. It allows the real-time observation of complexes with single-molecule resolution and has the potential for a high-throughput format [48–50]. While standard microarray scanners that are equipped for dual wavelength detection suffice for most analyses, live imaging microscopy is needed for time point measurements [44,51]. More recently, quantitative analyses have become feasible. For example, the dissociation constant of the interaction between SUMO1 and Ubc9 was determined. The results show good consistency with measurements done with other methods, which are well established as being quantitative, such as surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) [48].

Conclusions
- Strengths: *in vivo* localization; high sensitivity.
- Weaknesses: potentially high background due to autofluorescence, low throughput.
- Limitations: detection range limited by spacer length.

Bimolecular fluorescence complementation

BiFC is a specific version of a protein complementation assay (Figure 2). It is based on the measurement of the signal intensity generated by a fluorescent protein that is recreated by a

reconstitution from two nonfluorescent protein fragments, which are attached to the proteins (bait and prey) under study. The fluorescence intensities can either be measured with a simple fluorometer or by cell cytometry, or directly be visualized in subcellular locations by fluorescence microscopy [43]. Some reports say that BiFC is superior to FRET in terms of dynamic range [52]. Systematic protocols exist for the development of split protein systems for PPI studies [53]. Although the degree of unspecific complementation of the nonfluorescent protein fragments does generate background signal, continuing improvements have established BiFC as a means of studying PPI in bacterial, plant and mammalian cell systems [54]. Many different fluorescent proteins with a wide range of fluorescent spectra are in use, such as green fluorescence protein in different variants, Dronpa, Venus and mCherry. Recently, a new version of the split Venus system exhibited rather little background interference and enabled the sensitive visualization of cofilin–actin and Ras–Raf interactions [55].

Conclusions
- Strengths: no expensive infrastructure needed; low background; high sensitivity (detection of weak and transient interactions).
- Weaknesses: stability of the reconstituted fluorophore complexes; nonspecific interactions.
- Limitations: limited detection of dynamic changes in PPIs.

Fluorescence correlation spectroscopy

FCS is a well-established method to characterize PPI in living cells with single-molecule sensitivity. Detection is by confocal microscopy. Fluorescently labeled molecules are excited in a very small detection volume (picoliters to femtoliters). The diffusion of the molecules in and out of the detection volume causes the fluorescence intensity to fluctuate randomly. FCS measures these fluctuations, thus yielding information about the motion of the molecules [45,56]. When a fluorescently labeled ligand binds to another molecule, its mobility will be restricted by the interaction partner, thus affecting the fluctuation rate. Even more accurate is fluorescence cross-correlation spectroscopy (FCCS) in which both interaction partners are labeled. A comprehensive review [45] describes both theory and technical developments of FCS in proteomics. The techniques applications for visualizing and measuring PPI were also reported in more detail elsewhere [56].

Conclusions
- Strengths: high specificity; very small sample volumes and concentrations (picomolar range); single molecule sensitivity.
- Weaknesses: loss of signal through photobleaching; low throughput.
- Limitations: stability of the fluorophore.

Proximity ligation

A more recent development for the intracellular detection of PPI is the proximity ligation assay [46]. The procedure requires the availability of specific and affine binder molecules that target the protein partners under investigation. At least two such binders – may they be antibodies, single-chain fragment binders, affibodies, aptamers, nanobodies, DARPins or other affinity reagents – are tagged with a specific oligonucleotide sequence each. Once incubated on tissue samples, for example, histological specimens, the two molecules will bind to their respective protein targets. If the binding sites are in close proximity, then the single-stranded oligonucleotides will interact, helped by a third DNA oligonucleotide that is complementary to the ends of both, and can be covalently connected by ligation. The resulting DNA is amplified via rolling circle amplification during which fluorescence dyes are incorporated, thus achieving single-molecule sensitivity when analyzed with a fluorescence microscope.

Conclusions
- Strengths: studying PPIs in tissue sections; high sensitivity.
- Weaknesses: no multiplexing capacity; needs highly specific antibodies.
- Limitations: no information on the direct interaction of two proteins (colocalization only).

Methods for relative PPI quantification

2D polyacrylamide gel electrophoresis

2D electrophoresis (Figure 2) is a powerful tool to identify interacting protein partners and to compare protein expression among different samples. Until the emergence of MS analyses, 2D PAGE formed the technical backbone of protein analysis. However, a high degree of variability and thus low reproducibility are major disadvantages, apart from the work intensive procedures involved. In a standard setup, a fractionated protein mixture (e.g., after immunoprecipitation) is separated according to isoelectric point in the first dimension and molecular weight in the second dimension. Individual protein spots are then extracted from the gel and digested into peptides, which are used for identification by MS. Despite attempts to automate processes for spot detection and alignment, the method is rather low throughput and time-consuming.

An adaptation of 2D electrophoresis called differential in gel electrophoresis is used to run up to two different samples plus an internal standard in the same gel. Each sample is labeled with a fluorescent dye of a distinct wavelength, which is covalently linked to the proteins. The method has a better reproducibility and dynamic range than traditional 2D PAGE analyses. With an internal standard (e.g., a pool made of all analyzed samples), the method becomes quantitative since protein abundances can be normalized [57]. Recently, electrophoresis at native conditions was applied to reveal multiprotein complexes in the cytoplasma of red blood cells [58]. For this approach, pre-enrichment is required, however, in order to achieve specificity.

Conclusions
- Strengths: cheap, high resolution using different pH ranges, use of crude extract possible.

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- Weaknesses: low throughput, low reproducibility, no multiplexing, time-consuming.
- Limitations: limited robustness.

Atomic force microscopy

Atomic force microscopy measures the interaction force between two individual proteins; one protein is immobilized on a solid support, while the other one is attached to the tip of the atomic force microscopy cantilever. The tip moves perpendicular to the solid support surface, measuring the force exerted to the attached protein. The binding partners are usually highly pure recombinant proteins. The method is useful for a relative comparison of interaction strengths, like for drug screening, during which protein interaction is measured in the presence of several drug candidates. The ability of cynarin to act as an immunosuppressor drug by blocking interaction between CD28 and CD80 was identified in this way, for example [59].

Conclusions

- Strengths: sensitive, kinetic measurement, atomic resolution.
- Weaknesses: low throughput, expensive equipment, small size of scanned images.
- Limitations: requires highly purified proteins.

Methods for the absolute quantification of PPIs

Binding affinity is defined as the strength of an interaction between two (or more) proteins in equilibrium. It is measured as dissociation constant (K_d), which is the concentration of the free protein at which it occupies half of the overall binding sites of the second protein [10,60]. Only methods defining a K_d are suitable for absolute PPI quantification [61]. Various analysis processes are around that allow such assays. On the one hand, there are label-free techniques such as ITC, bio-layer interferometry (BLI), backscattering interferometry (BSI), circular dichroism (CD) and analytical ultracentrifugation [62,63]. Other processes are based on fluorescence spectroscopy such as microscale thermophoresis (MST) or fluorescence polarization [45]. NMR spectroscopy [12] forms a third group, generating structural details of protein interactions at an atomic resolution.

Label-free methods to study PPI

Isothermal titration calorimetry

ITC is a label-free proteomic technology that provides a real-time kinetic measurement of PPIs by determining the heat uptake or release during an interaction. Besides analyzing the binding affinity, also the enthalpy, entropy and change in heat capacity of an interaction are determined. Reaction rates of up to 10^{-12} mol/s can be handled and the thermosensitivity is high since changes as small as 0.1 µcal (0.4 µJ) can be detected [64,65]. PPIs of very high or low affinity may not be described fully quantitatively, however. Although a relatively new method, acceptance by the scientific community has been good and led to its frequent application [66].

Conclusions

- Strengths: accurate kinetic values and stoichiometry of an interaction in a single experiment; high sensitivity; label-free detection.
- Weaknesses: high sample consumption; time-consuming; low throughput; expensive equipment is required.
- Limitations: highly purified proteins are needed.

Backscattering interferometry & bio-layer interferometry

BSI measures the change of the refractive index that results from the interaction of two molecules. It facilitates analyses in solution and has picomolar sensitivity for the quantification of binding affinities as association and dissociation rate constants [20]. For example, the dissociation constants of α-crystallin chaperone interactions were analyzed in picoliter sample volumes [67]. Other studies aimed at molecule pairs such as protein A and IgG, IL-2 and a related monoclonal antibody, calmodulin and calcium ions, or the protein calcineurin and the M13 peptide [20].

BLI is a plate-based real-time optical process that detects the shift in the interference patterns of light, which is reflected from two different layers [68]. Kinetic rates and affinity constants can be determined in a quality that is comparable to SPR but is less time-consuming because of the array format [69]. One application was a screen of the potential of 6500 compounds to affect specifically and significantly the interaction of three human protein pairs including BCL-2 and eIF4E [70]. The kinetic mechanisms proposed for the enzyme–substrate complex of cJun-terminal kinase (JNK1β1) and its two substrates are based on BLI measurements [71].

Conclusions

- Strengths: small sample volume; label free (BSI and BLI); compatible with crude samples (BSI and BLI); no surface attachment optimization (BSI), high throughput (BLI).
- Weaknesses: special infrastructure is required; low-throughput method (BSI).
- Limitations: highly specific protein attachment is prerequisite (BLI).

Circular dichroism

Another label-free method in proteomics is based on the effect of CD. CD is the difference in absorption by chromophores of left and right circularly polarized light. Since the interaction of a protein with a ligand or another protein affects the protein conformation, the CD spectrum does shift. This can be examined at various concentrations of the binding partner at different time points, thereby providing information about the binding constant and thermodynamics [72]. The analysis of PPI by CD spectroscopy requires protein of high purity, however, and is only suitable for completely dissolved proteins [63]. The technology has been used successfully, for instance, for determining the function of weak interactions of protein kinase R dimerization as dsRNA-binding domain [73]. The role of single

C-terminal mutations of p53 in changing the affinity to MDM2 was also examined with CD spectroscopy [74].

Conclusions
- Strengths: label-free detection; high specificity; quick assay; measures kinetics.
- Weaknesses: low throughput; high sample consumption; expensive equipment; labor intensive.
- Limitations: low structural resolution; low sensitivity to structural changes.

Surface plasmon resonance

SPR (Figure 2) is an affinity-based optical technique that enables real-time detection and quantification of biomolecular interactions on a gold surface. Binding of molecules in solution to surface-immobilized molecules alters the refractive index of the medium near the surface. In order to analyze a binary interaction, one protein is immobilized onto the sensor surface, and its binding partner is added to the aqueous solution. As the analyte binds to the immobilized protein, the accumulation of molecules on the surface results in altering the angle at which polarized light is reflected. During dissociation, the inverse effect is observed. The change in angle is recorded from which kinetic constants can be retrieved and mass proportions be calculated. SPR is currently the most commonly used method for characterizing the kinetics of PPI [75], and the use of SPR coupled to MS has been reported [76,77]. Similar to the other methods, for which one protein interaction partner is immobilized, this can potentially lead into a loss of function/activity due to the loss of the protein's 3D structure.

Conclusions
- Strengths: label-free detection; real-time kinetic measurement; no need for purified sample; can be coupled to MS.
- Weaknesses: low throughput; expensive equipment; expertise required for data analysis.
- Limitations: not suitable for detection of low-molecular weight molecules; mass transport can affect kinetic analysis.

Nuclear magnetic resonance

NMR encompasses different useful techniques to measure a very wide range of dissociation constants (from 10^{-6} to 10^{-2} M). The most widely used NMR technique to study PPIs is chemical shift perturbation mapping, in which the ^{15}N-^{1}H heteronuclear single quantum coherence spectrum of an ^{15}N-labeled molecule allows to follow shifts of the amino acid resonance upon addition of an unlabeled partner [78]. Another technique, in principle similar to FRET, relies on the Nuclear Overhauser effect to measure the distance between two protons, thus using spatial information for the identification of interaction [79]. One-dimensional or 2D NMR spectra allows titrations of one compound relative to a partner, using analysis techniques adapted to either slow, intermediate or fast reactions. A large number of other NMR methods exist to study protein–protein contacts and even the dynamics of the protein backbone by making use of relaxation times (reviewed in [80]).

Conclusions
- Strengths: measures broad dynamic ranges of PPI.
- Weaknesses: very high sample consumption; expertise and very expensive equipment required; labor intensive; internal protein labeling required.
- Limitations: only suitable for small and soluble proteins.

Fluorescence-based analyses

Microscale thermophoresis

Two types of fluorescence spectroscopy methods are widely used to detect and study PPIs. MST is based on the thermophoretic effect, which refers to the motion of fluorescently labeled molecules in a microscopic temperature gradient. Any change to the conformation and therefore a molecule's hydrate shell results in a change of the movement along the gradient. These data are used to determine binding affinities with low sample consumption [81]. The method allows measurements of protein interactions in not only artificial buffer systems but also body liquids or cell lysates under near-native conditions. For example, the interaction of the small-molecule inhibitor quercetin and the cAMP-dependent kinase (PKA) exhibits a binding constant in human serum that is 400-fold lower than that in an analysis buffer. The simplicity of sample preparation for MST analyses provides an opportunity to reveal such differences [82]. Similarly, it has been shown that the method is highly sensitive to all kinds of changes of molecular properties such as size and charge [83].

Conclusions
- Strengths: measures binding affinities; no purified protein needed; low sample consumption; measurements possible under 'natural' conditions (body liquids, cell lysates).
- Weaknesses: highly sensitive to all kinds of changes of molecular properties such as size and charge.
- Limitations: low specificity, high background.

Fluorescence polarization or anisotropy spectroscopy

When fluorophores are exposed to polarized light, the emission is also polarized, an effect termed anisotropy. The polarized emission is affected by the motion of a fluorescence dye and thus the tumbling of the molecule to which the fluorophor is attached. Consequently, the signal is influenced by the size and shape of a molecule [45]. A protein interaction is therefore bound to change the spectrum by altering indirectly the movement of the fluorophor attached to the protein. However, also additional parameters have to be considered, such as the variation of the viscosity of the solvent, for instance. Monitoring and quantification occur in homogenous solution and real time [84,85]. In one study, a rhodamine-labeled p53 peptide was used to identify the effect that small molecules have on the interaction of p53 with MDM2 and MDM4 under various

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conditions [86]. Upscaling of the throughput was implemented by means of a microfluidic system with picoliter droplets and used to study the interactions between proteins and related antibodies [85].

Conclusions

- Strengths: measurements in real time; high dynamic range and temporal resolution.
- Weaknesses: expensive infrastructure; stability of the fluorophore in the excited state.
- Limitations: the signal is influenced by the size and shape of a molecule.

Mass spectrometry

Mass spectrometers are made up by three main components in which the following processes take place: ionization; ion separation based on a mass to charge ratio (m/z); and ion detection [87]. Originally, size and polarity of proteins had been a problem to MS that was mainly used for the analysis of small molecules. This obstacle was overcome when electron spray ionization and matrix-assisted laser desorption ionization were introduced [88,89]. Currently, MS represents the gold standard for many forms of analysis in proteomics. Generally, MS is done with proteins that are partially purified by electrophoresis or chromatography techniques, enriched or fractionated and subsequently digested using a proteolytic enzyme. Numerous approaches of front end separation techniques were developed to improve the accuracy, efficiency and sensitivity of detection in mass spectrometers [87]. The enrichment of low-abundance proteins or the depletion of highly abundant proteins is important to increase the relative number of proteins of interest; an obvious example is the depletion of albumin from plasma samples [90]. Since mass variations are analyzed, MS is able to analyze protein modifications (e.g., the phosphoproteome). More recently, also applications for protein quantification have become possible [87]. Relative quantification is performed by comparing two or more protein samples, either using stable isotope labeling or label-free tagging strategies. When internal, well-characterized peptide standards are available, then even an absolute quantification is feasible by comparing peak sizes [91,92].

Labeling strategies for quantification

Isotopic labeling of proteins for relative quantification is done through enzymatic, metabolic or chemical processes. Enzymatic labeling of peptides with ^{18}O is performed during the proteolytic digestion with trypsin [61]. The most frequent metabolic labeling strategy is stable isotope labeling of amino acids in cell cultures. This *in vivo* technique takes advantage of heavy or light amino acids, typically lysine and/or arginine, as supplement in the culturing medium or the animal diet [93]. The method is applicable to proteins derived from cell cultures as well as samples from animal's body fluids or tissues [91,93,94]. For the latter or human samples, *in vitro* chemical labeling is an alternative. Generally, the chemical labeling reagents contain a reactive group to couple to proteins or peptides, a known mass tag or a functional molecule for capturing and separation [61]. The two most popular techniques for isotopic labeling are isotope-coded affinity tags (ICATs) and isobaric tags. The ICAT reagent contains iodoacetamide groups that conjugate isotopic hydrogen or carbon atoms and a biotin tag to cysteines [91,95]. After labeling, proteins from two samples are combined and digested. Then, biotin AP is performed to enrich labeled peptides prior to the MS analysis. Recently, a cleavable biotin group was developed for an improvement of the post purification steps [61]. In the latest generation of this method, a visible and photocleavable ICAT is used to directly monitor labeled peptides during the separation step; it also has been used for absolute quantification [96]. The isotope-coded protein label techniques [61] add stable isotope tags at the free amino groups of intact proteins. In particular, isobaric tags are commonly used [97] because they allow multiplex experiments [87].

Label-free quantification

Although isotopic labeling strategies continue to dominate, label-free MS techniques are making progress [23,98]. In reference to the shotgun approach in genomic sequencing, the term 'shotgun proteomics' was coined to describe quantitative, label-free, MS-based proteomics [99]. Shotgun proteomics relies on front end fractionation techniques such as isoelectric focusing, liquid chromatography (LC) and reverse phase chromatography [90,99]. Relative quantification is simply done by directly comparing the tryptic peptides contained in protein samples. The peptides from complex samples are separated by a combination of chromatographic techniques and usually tandem MS (MS/MS). The resulting MS fingerprints made up by the peptide peaks are compared with databases for an identification of the proteins from which the peptides originate.

The latest LC–MS/MS or LC/LC–MS/MS processes can generate several peptide indicators to determine protein abundances [91]. The two common categories of label-free quantification are based on peak intensity (chromatogram) at a particular retention time and spectral counting approaches [99]. A recent study reports that a combination of peak intensity and spectral counting significantly increases the number of proteins for which abundance differences can be detected [100]. Data normalization and analysis are challenging features of label-free proteomics. Numerous software analysis tools and databases have been developed for this purpose such as Decyder MS, accurate mass tag, Mascot and others [61].

Absolutely quantitative proteomics

The best-established MS-based method for quantification is multiple selected reactions monitoring. This process takes advantage of triple quadrupole mass analyzers that perform consecutively a mass selection, fragmentation and mass detection [101]. Multiple proteins can be monitored in a single MS run. Selected proteotypic peptides of known fragmentation characteristics stand for particular proteins of interest. These peptides are compared with a dilution of standard peptides, frequently produced by chemical synthesis, which are labeled with

a stable isotope and act as an internal reference when spiked into the samples in known quantities. Alternatively, artificial concatemer peptides can be expressed in bacteria using metabolic labeling. Also complete proteins have been used as standard [61,101]. In extension to multiple selected reactions monitoring, parallel reaction monitoring yields quantitative data over a wider dynamic range since not just one, but all peptides produced during fragmentation are monitored [102]. For a computational prediction of suitable proteotypic peptides, several algorithms have been developed, which select specific peptides on the basis of known physiochemical protein properties [103,104].

Combination assays

For all their potential, MS techniques are frequently applied in combination with other procedures of proteome analysis. Most widely used methods that are paired with MS are AP, cross-linking, immunoprecipitation and SPR [18,22,23,105]. AP–MS techniques were designed that preserve labile interaction in protein complexes [106]. Furthermore, stable isotope labeling of amino acids in cell cultures labeling is very compatible with AP–MS to monitor and map PPIs intracellularly [21], as utilized for a comparison of the interaction mechanism of the protein phosphatase and tensin homolog in normal and cancer cells [107] or the identification of the interaction partners of β-catenin and the E3 ubiquitin–protein ligase Cbl [108], for example.

To address the problem of detecting transient protein interactions, several chemical cross-linking techniques are being employed and the detected PPIs quantified with MS-based analyses. The choice of a cross-linker reagent depends on several factors such as its cell-permeability, reactivity and spacer length [106]. In various transient protein interaction studies, the combination of chemical cross-linking and LC–MS has identified abundance level of changes in protein–peptide interactions [18]. A novel category of cross-linking is embodied by the protein interaction reporter (PIR) technology. Unlike traditional chemical cross-linkers, the PIR cross-link can be specifically cleaved *in situ* to release two intact peptide chains in MS. PIR/cross-linking MS with its unique attributes holds great potential for mapping PPIs on a large scale [109].

Expert commentary & five-year view

Proteins execute many biological functions in a cell, and many regulatory processes take place at the protein level. Consequently, 97% of current therapeutic agents target proteins. In addition, the ability to sequence the information encoded in nucleic acids at a comprehensive scale spills over into proteomics, providing lots of basic information about variations that are translated and thus relevant at the protein level. Simultaneously, the limitations of an isolated genome analysis become even more apparent, in particular, for biomedical purposes. The obvious central biological role of proteins and the requirement of following up in the proteome, many of the initial results obtained at the genome level has promoted proteomic studies to the next pillar of a comprehensive molecular analysis. In order to achieve this task, it is likely that a number of different techniques, and combinations thereof, will be required. However, even some basic reagents, such as a set of specific and affine binder molecules that target the entire basic set of gene products, are missing or just in the making, and many biochemical aspects are kind of neglected for the lack of appropriate analysis techniques.

As part of the above, the aspect of protein interaction is crucial. Many interactions are needed to achieve or regulate an activity. Others have to be tolerated by a protein in order to execute its task although proteins or ligands are around; it is unlikely that the environment in a cell allows any molecule to perform its activity in isolation. The development of methodologies has made substantial advances toward high throughput and quantification. However, a combination of quantification and really comprehensive coverage of a proteome is still not possible, not least because of the complexity that needs to be dealt with. In addition, the variations that occur over time in response to other changes are not tackled in a comprehensive manner, but are nevertheless required for modeling the interactions and the resulting functions. Therefore, this field of proteomics is still at its beginning, irrespective of its impressive advances recently. And given the task ahead, it may well take another decade or two before analyses can be performed that are similar in coverage and accuracy to what is now possible at the level of nucleic acids.

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Key issues

- Protein–protein interactions (PPIs) are at the heart of almost all biological processes and present prime targets for therapeutic intervention.
- To study PPIs, there is a variety of techniques available offering either high-throughput analysis or absolute quantification.
- To fully appreciate the complexity of cellular proteomic interactions, new technologies are needed that integrate both these aspects.
- Once accomplished, these technologies will boost our understanding of PPIs, yielding new therapeutic targets and approaches.

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Notice of correction

The order of the authors in the author list on page 1 was changed post publication.