EXPERT REVIEWS

Interactomics: toward protein function and regulation

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Shachuan Feng[‡1,2], Li Zhou[‡1,2], Canhua Huang[3], Ke Xie*[1] and Edouard C Nice*[3,4]

[1]*Department of Oncology, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, Chengdu, 610072, PR China*
[2]*School of Life Sciences, Sichuan University, Chengdu, 610065, PR China*
[3]*The State Key Laboratory for Biotherapy/ Collaborative Innovation Center of Biotherapy, West China Hospital, Sichuan University, Chengdu, 610041, PR China*
[4]*Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, 3800, Australia*
**Authors for correspondence:*
Tel.: +86 13008154897
Fax: +61 399 029 500
ed.nice@monash.edu
mei97@sina.com

[‡]*These authors contributed equally to this work*

Protein–protein interactions are central to all cellular processes. Understanding of protein–protein interactions is therefore fundamental for many areas of biochemical and biomedical research and will facilitate an understanding of the cell process-regulating machinery, disease causative mechanisms, biomarkers, drug target discovery and drug development. In this review, we summarize methods for populating and analyzing the interactome, highlighting their advantages and disadvantages. Applications of interactomics in both the biochemical and clinical arenas are presented, illustrating important recent advances in the field.

KEYWORDS: interactomics • network analysis • network hubs • protein–protein interactions • proteomics

For many years, scientists have mainly studied one gene at a time in isolation from the larger context of other genes. The decoding of the human genome in 2001 opened the way for analysis of common features in the genome as well as genetic variations among individual humans [1]. Since then, rapid advances in genomic studies are facilitating the understanding of life processes through investigation of gene expression and function. Concurrently, the development of technologies such as microarrays has allowed genome-wide analysis of transcriptomes [2]. However, to translate this knowledge into practical benefits through a deeper understanding of biological systems, new 'omics' techniques were required to enable the generation of large-scale inventories of the proteins expressed in cells (the proteome), including clarification of how genome-encoded products interact in complex networks (the interactome), and how genetic variation can modify the structural and dynamic behavior of these networks. Although the proteome is encoded by the human genome, many features of proteins, from their structure and function to their disease-related modifications, cannot directly be deduced from genomic analysis. To assist this, the Human Proteome Organization was established to help accelerate proteomic research through development of required technologies and standards, training and education of investigators in proteomic sciences and facilitation of multidisciplinary international collaborations [3,4]. Three 'pillars' to support this endeavor have been established: mass spectrometry (MS), antibody-based approaches and knowledge-based systems [5]. Proteomic studies can reveal the role of proteins in pathological processes, thus facilitating the early diagnosis of disease, prognosis prediction, identification of new therapeutic targets and evaluation of treatment response [6]. As it will soon be possible to sequence an intact human genome for less than US$ 1000 in a fraction of an hour, the combination of genomics, proteomics and transcriptomics will help realize the goal of personalized medicine [7]. However, because protein interactions are fundamental to structuring and regulating essentially all biological processes, interactomics studies are required to progress such studies. Interactomics is a fusion of biochemistry, informatics and engineering, which provides a global view of protein interaction networks. Mapping the interaction networks of an organism will contribute to understanding the pathophysiology of diseases and defining new biomarkers and drug targets. In addition, analysis of the interactome will reveal functional associations in cellular networks. In this review, we will discuss available methods for detection, validation and analysis of the interactome and highlight recent applications that have advanced the field.

How do we populate the interactome?

Proteins play important roles in most biological processes, and their interactions with each other precisely regulate biological function. Therefore, populating and understanding

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specific interactomes is becoming extremely important. A number of approaches have been developed in recent years to detect protein–protein interactions (PPIs). These approaches can be roughly divided into three groups: *in silico*, *in vivo* and *in vitro* [8]. Each group includes many different technologies. The *in silico* methods, which consist of text mining and computational analyses, are usually carried out by computer simulation. *In vivo* methods include yeast two-hybrid (Y2H), protein-fragment complementation assay (PCA) and mammalian protein–protein interaction trap (MAPPIT) and can be performed on intact living organisms. In the case of *in vitro* methods, the experiments are performed in a controlled environment outside a living organism, and include methods such as tandem affinity purification-mass spectroscopy (TAP-MS), protein microarray and the luminescence-based mammalian interactome (LUMIER) technique. In addition, we will introduce emerging novel methods for the study of PPI interfaces that aid in deciphering the mechanisms of PPI.

In silico *methods for the prediction of PPIs*

All of the methods used to detect PPIs have their own advantages and disadvantages. Therefore, it is important to populate PPIs using more than one approach for both confirmation and validation in order to avoid false-positive results. Since high-throughput techniques for the experimental determination of PPIs are still relatively limited, *in silico* methods for detecting PPIs from protein interaction data are complementary to such studies [9]. Additionally, they enable a systematic analysis of the experimental data, as well as the prediction of novel PPIs and the planning of experimental studies for their detection.

Text mining can be properly applied to collect interaction data by searching the available literature to find out PPIs that have been reported from low-throughput protein interaction studies [10]. This approach is done either manually or can be automated using criteria such as co-occurrence of gene or protein names in publication abstracts, or applying more sophisticated semantic algorithms [11]. Available public databases include the Munich Information Center for Protein Sequence [12], the Molecular Interaction database [13], the Human Protein Reference Database [14], bioGRID [15], CREDO [16] or IntAct (Tables 1 & 2) [17].

In addition to text mining, a variety of computational methods have been developed to supplement the PPI data that have been determined experimentally. There are three basic approaches for the analysis of experimental PPI data, namely the ortholog-based approach, the structure-based approach and the co-evolution analysis approach.

Ortholog-based approach

This approach is based on finding orthologs for all available genes and their organization in the genome to construct hypotheses on the functional relationships of a range of genes [18]. Several proteins from an organism under study may share significant similarities with proteins involved in complex formation in other organisms [8]. Such proteins can be further classified into orthologs and paralogs. However, these are two totally different concepts used in the prediction process. Orthologs will retain functionality during the course of evolution, whereas paralogs may acquire new functions. Thus, if two proteins A and B interact with each other, then the orthologs of A and B in a new species are also likely to interact with each other.

Structure-based approach

Due to the rapid growth in the number of protein structures that have been determined, structure-based methods which can predict possible protein–protein interaction if two proteins have a similar structure are currently gaining importance [19]. If the interacting domains of two proteins can be established from their 3D protein structures, it is possible that other proteins that possess similar structure may also interact with each other through this region. Additionally, it has been reported by several groups through testing of 3D protein structures that binding affinities are associated with interface areas of PPIs [20–22]. These studies showed how calculated biophysical features correlated with binding affinity and determined the features that could be used to distinguish between high-, medium- and low-affinity PPIs. Thus, this approach can also be used for PPI prediction. However, many proteins do not have known structures. In this case, it is necessary to search the PDB database to allow simulation of the structure of a query protein based on sequence homology [23]. This method is available online (InterPreTS: Table 2) and enables the identification of homologous protein pairs from a database of interacting domains that contains structures of known protein complexes [24]. Structural prediction methods can be evaluated and assessed using the critical assessment of structure prediction [25].

Co-evolution analysis approach

Another important method for detection of protein–protein interactions is co-evolution analysis. This method is based on evolutionary relationships and can be used to detect PPIs or functional relationships of proteins from their phylogenetic profiles [26]. The concept is that if two proteins have a functional linkage in a genome, there will be a strong pressure on their orthologs to be inherited together during the evolution process [27]. Thus, it is possible to detect the presence or absence (co-occurrence) of proteins in the phylogenetic profile, because proteins having similar profiles will be functionally connected [28]. However, there are limitations with this method. Many unexpected disturbances caused by genomic events, such as gene duplication or loss could take place during co-evolution that would subvert the phylogenetic profile of single genes. This method appears more suitable for prokaryotes than eukaryotes [29].

In vivo *methods for the detection of PPIs*

While predicting interactions using *in silico* approaches can be useful, analysis of the biological system is required to reach definitive conclusions. Methods for *in vivo* detection of PPIs

Table 1. Protein–protein interaction databases.

Database	Description	Ref.
APID (Agile Protein Interaction Data Analyzer)	APID is an interactive bioinformatic web tool that has been developed to allow exploration and analysis of main currently known information about protein–protein interactions integrated and unified in a common and comparative platform	[130]
Binding DB	BindingDB is a public, web-accessible database of measured binding affinities, focusing chiefly on the interactions of protein considered to be drug targets with small, drug-like molecules. BindingDB contains 1,058,945 binding data, for 6997 protein targets and 453,657 small molecules	[131]
Binding Interface Database (BID)	Binding Interface Database collects numerous biomolecular interfaces	[132]
BioGRID	BioGRID is an online interaction repository with data compiled through comprehensive curation efforts	[133]
Biomolecular Interaction Network Database (BIND)	BIND stores descriptions of molecular complexes and pathways and biomolecular interactions	[134]
Biomolecular Object Network Databank (BOND)	New resource to perform cross-database searches of available sequence, interaction, complex and pathway information integrates a range of component databases including GenBank and BIND, the Biomolecular Interaction Network Database	[135]
Conserved Domain Database (CDD)	A collection of sequence alignments and profiles representing protein domains conserved in molecular evolution. It also includes alignments of the domains to known 3D protein structures in the MMDB database	[136]
CREDO	CREDO is a unique relational database storing all pairwise atomic interactions of inter- as well as intramolecular contacts between small molecules and macromolecules found in experimentally determined structures from the Protein Data Bank	[137]
CYGD	PPI section of the comprehensive yeast genome database	[138]
Database of Interacting Proteins (DIP)	The DIP database catalogs experimentally determined interactions between proteins, which were curated, both, manually by expert curators and also automatically using computational approaches. It combines information from a variety of sources to create a single, consistent set of protein–protein interactions	[139]
DOMINE	DOMINE is a database of known and predicted protein domain (domain–domain) interactions. It contains interactions inferred from PDB entries, and those that are predicted by 13 different computational approaches using Pfam domain definitions	[140]
DOMINO	DOMINO is an open-access database comprising more than 3900 annotated experiments describing interactions mediated by protein interaction domains	[141]
H-Invitational Database (H-InvDB)	By extensive analyses of all human transcripts, this database provide curated annotations of human genes and transcripts that include gene structures, alternative splicing variants, non-coding functional RNAs, protein functions, functional domains, subcellular localizations, metabolic pathways, protein 3D structure, genetic polymorphisms (SNPs, indels and microsatellite repeats), relation with diseases, gene expression profiling and molecular evolutionary features, protein–protein interactions and gene families/groups	[142]
HIV-1, Human Protein Interaction Database	A database of known interactions of HIV-1 proteins with proteins from human hosts. It provides annotated bibliographies of published reports of protein interactions, with links to the corresponding PubMed records and sequence data	[143]
Homomint	Homomint is a web available tool extending protein–protein interactions experimentally verified in models organisms, to the orthologous proteins in Homo sapiens	[144]

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Table 1. Protein–protein interaction databases (cont.).

Database	Description	Ref.
HUGE ppi	HUGE ppi is a database of protein–protein interactions between large proteins	[145]
Human Annotated and Predicted Protein Interactions (HAPPI)	HAPPI database v2.0 contains 640,798 physical and functional human protein–protein interactions, perfect for network biology and network medicine applications	[146]
Human Protein Reference Database	A platform to visually depict and integrate information pertaining to domain architecture, post-translational modifications, interaction networks and disease association for each protein in the human proteome	[147]
IntAct	IntAct provides a freely available, open source database system and analysis tools for protein interaction data. All interactions are derived from literature curation or direct user submissions and are freely available	[148]
Inter-Chain Beta-Sheets (ICBS)	ICBS is a database of protein–protein interactions mediated by interchain β-sheet formation	[149]
Interologous Interaction Database (I2D)	I2D is an online database of known and predicted mammalian and eukaryotic protein–protein interactions	[150]
Kinetic Data of Biomolecular Interactions (KDBI)	KDBI is a collection of experimentally determined kinetic data of protein–protein, protein–RNA, protein–DNA, protein–ligand, RNA–ligand, DNA–ligand binding or reaction events described in the literature	[151]
neXtProt	neXtProt seeks to be a comprehensive human-centric discovery platform, offering its users a seamless integration of and navigation through protein-related data	[152]
PDZBase	PDZBase is a manually curated protein–protein interaction database developed specifically for interactions involving PDZ domains. PDZBase currently contains 339 experimentally determined protein–protein interactions	[153]
PepCyber: P~Pep	Database of human protein–protein interactions mediated by phosphoprotein binding domains	[154]
PeptideAtlas	The long-term goal of the PeptideAtlas project is full annotation of eukaryotic genomes through a thorough validation of expressed proteins. The PeptideAtlas provides a method and a framework to accommodate proteome information coming from high-throughput proteomics technologies	[155]
PRIDE Archive	This is a centralized, standards compliant, public data repository for proteomics data, including protein and peptide identifications, post-translational modifications and supporting spectral evidence	[156]
ProNIT	ProNIT database provides experimentally determined thermodynamic interaction data between proteins and nucleic acids	[157]
Protein Clusters	Protein Clusters is a database of proteins grouped together by sequence similarity	[158]
Protein Database	A database that includes protein sequence records from a variety of sources, including GenPept, RefSeq, Swiss-Prot, PIR, PRF and PDB	[159]
ProteomeXchange	This database provides a coordinated submission of MS proteomics data to the main existing proteomics repositories, and to encourage optimal data dissemination	[160]
PSIbase	PSIbase focuses on structural interaction of proteins and their domains, and is based on PSIMAP that is a map of protein interactome, and it covers the interaction of all known 3D protein structures	[161]
SPIDer	SPIDer is a database for a predicted protein–protein interaction dataset which is solely derived from the Gene Ontology (GO), based on the genome of yeast *Saccharomyces cerevisiae*, and provides users with a graphical interface for visualizing an interaction subnetwork for a list of proteins of interest	[162]

Table 1. Protein–protein interaction databases (cont.).

Database	Description	Ref.
STRING	STRING is a database of known and predicted protein interactions. The interactions include direct (physical) and indirect (functional) associations; they are derived from four sources: Genomic Context, High-throughput Experiments (Conserved) Co-expression, Previous Knowledge	[163]
The UniProt Knowledgebase (UniProtKB)	This is the central access point for extensive curated protein information, including function, classification and cross-reference, and consists of Swiss-Prot, the manually annotated section, and TrEMBL, the computationally analyzed section	[164]
The Global Proteome Machine Database (GPMDB)	The GPMDB was constructed to utilize the information obtained by GPM servers to aid in the difficult process of validating peptide MS/MS spectra as well as protein coverage patterns	[165]
The Human Protein Atlas	The Human Protein Atlas portal is a publicly available database with millions of high-resolution images showing the spatial distribution of proteins in 44 different normal human tissues and 20 different cancer types, as well as 46 different human cell lines	[166]
The Human Protein Interaction Database (HPID)	HPID was designed to provide human protein interaction information pre-computed by a statistical method from existing structural and experimental data, to provide integrated human protein interactions derived from BIND, DIP and HPRD and to find proteins from the databases that potentially interact with proteins submitted by users	[167]
The Human Protein Reference Database (HPRD)	HPRD represents a centralized platform to visually depict and integrate information pertaining to domain architecture, post-translational modifications, interaction networks and disease association for each protein in the human proteome	[168]
The MIPS Mammalian Protein–protein Interaction Database	The MIPS Mammalian Protein–protein Interaction Database is a collection of manually curated high-quality protein–protein interaction data collected from the scientific literature by expert curators	[169]
The Molecular INTeraction database (MINT)	The MINT focuses on experimentally verified protein–protein interactions mined from the scientific literature by expert curators	[170]
The ProLinks database	The ProLinks database is a collection of inferences of functional linkages between proteins using 4 methods	[171]
The Protein Interaction and Molecule Search (PRIMOS) database	The PRIMOS database operates as an integrated knowledge portal for analyzing protein–protein interaction data	[172]
The Unified Human Interactome (UniHI)	The UniHI is a comprehensive database of computational and experimental based human protein interaction networks. This database is aimed to integrate diverge maps, which offers the researcher a flexible and direct entry gate into the human interactome	[173]
Three-Dimensional Interacting Domains (3did)	The database of 3did is a collection of high-resolution 3D structural templates for domain–domain interactions	[174]

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Table 2. Protein–protein interaction analysis software/online servers.

Software	Description	Ref.
ADAN (EMBL)	Prediction of protein–protein interAction of moDular domAiNs	[175]
Advanced Network Analysis Tool (ANAT)	ANAT is a tool for constructing and analyzing functional protein networks	[176]
ADVICE	Automated Detection and Validation of Interaction by Co-Evolution	[177]
ANCHOR	ANCHOR is a web server and database for analysis of protein–protein interaction binding pockets for drug discovery	[178]
APID (Agile Protein Interaction Data Analyzer)	APID is an interactive bioinformatic web tool that has been developed to allow exploration and analysis of main currently known information about protein–protein interactions integrated and unified in a common and comparative platform	[130]
BindN	BindN applies support vector machines (SVMs) to predict DNA- and RNA-binding residues from sequence features, including the side chain pKa value, hydrophobicity index and molecular mass of an amino acid	[179]
BioLayout Express^{3D}	BioLayout Express^{3D} is a powerful tool for the visualization and analysis of network graphs in 2D and 3D space derived primarily, but not exclusively, from biological data	[180]
Biologic Interactions and Network Analysis (BIANA)	BIANA is a web-based interface for PPI predictions based on interolog information	[181]
CAPER 2.0	An interactive, configurable and extensible workflow-based platform to analyze datasets from the Chromosome-centric Human Proteome Project	[182]
CAPIH	CAPIH is a Web interface for comparative analyses and visualization of host-HIV protein–protein interactions	[183]
catRAPID	catRAPID is an algorithm to estimate the binding propensity of protein-RNA pairs	[184]
Consensus Protein–protein Interaction Site Predictor (cons-PPISP)	cons-PPISP is a consensus neural network method for predicting protein–protein interaction sites	[185]
Conserved Domain Architecture Retrieval Tool (CDART)	Displaying the functional domains that make up a given protein sequence. It lists proteins with similar domain architectures and can retrieve proteins that contain particular combinations of domains	[186]
Conserved Domain Search Service (CD Search)	Identifying the conserved domains present in a protein sequence, CD-Search uses RPS-BLAST (Reverse Position-Specific BLAST) to compare a query sequence against position-specific score matrices that have been prepared from conserved domain alignments present in the Conserved Domain Database (CDD)	[187]
DBS-PRED	This program predicts the DNA-binding in a protein from their sequence information	[188]
DISPLAR	DISPLAR is a neural network method. Given the structure of a protein known to bind DNA, the method predicts residues that contact DNA	[189]
DNA-Binding Domain Hunter (DBD-Hunter)	DBD-Hunter is a knowledge-based method for predicting DNA-binding proteins function from protein structure. The method combines structural comparison and evaluation of DNA–protein interaction energy, which is calculated use a statistical pair potential derived from crystal structures of DNA–protein complexes	[190]
DP-Bind	DP-Bind is a web server for sequence-based prediction of DNA-binding residues in DNA-binding proteins	[191]
DroPNet	DroPNet is a web portal for integrated analysis of Drosophila protein–protein interaction networks	[192]
Dtome	DTome is a web-based tool for drug-target interactome construction	[193]

Table 2. Protein–protein interaction analysis software/online servers (cont.).

Software	Description	Ref.
FunFOLDQA	FunFOLDQA is a quality assessment tool for protein-ligand binding site residue predictions	[194]
Gene Interaction Miner (GIM)	GIM is a new tool for data mining of contextual information for protein–protein interaction analysis	[195]
Genes2Networks	Connecting lists of gene symbols using mammalian protein interactions databases	[196]
GPS-Prot	GPS-Prot is a web-based visualization platform for integrating host-pathogen interaction data	[197]
GraphCrunch 2	GraphCrunch 2 is a software tool for network modeling, alignment and clustering	[198]
Gold Reference dataset constructor from Information on Protein complexes (GRIP)	GRIP is a web-based system for constructing Gold Standard datasets for protein–protein interaction prediction	[199]
gsGator	gsGator is a web-based platform for functional interpretation of gene sets with useful features such as cross-species GSA, simultaneous analysis of multiple gene sets and a fully integrated network viewer for visualizing both GSA results and molecular networks	[200]
GUILDify	GUILDify is a web server for phenotypic characterization of genes through biological data integration and network-based prioritization algorithms	[201]
HotPoint	HotPoint predicts hotspots in protein interfaces using an empirical model	[202]
Human Annotated and Predicted Protein Interactions (HAPPI)	HAPPI database v2.0 contains 640,798 physical and functional human protein–protein interactions, perfect for network biology and network medicine applications	[146]
Human Protein Interaction Database (HPID)	HPID allows the user to use the protein IDs in ENSEMBL, HPRD and UniProt/Swiss-Prot ID to search protein interactions of interest	[167]
iELM	iELM is a web server to explore short linear motif-mediated interactions	[203]
Integrated Interactome System (IIS)	IIS is a web-based platform for the annotation, analysis and visualization of protein–metabolite–gene–drug interactions by integrating a variety of data sources and tools	[204]
iLIR	iLIR is a web resource for prediction of Atg8-family interacting proteins	[205]
IM Browser	IM Browser is both a database and a tool for exploring and integrating emerging gene and protein interaction data for Drosophila	[206]
Inferred Biomolecular Interaction Server (IBIS)	IBIS is a web server to analyze and predict protein interacting partners and binding sites	[207]
IntAct	IntAct provides a freely available, open source database system and analysis tools for molecular interaction data. All interactions are derived from literature curation or direct user submissions and are freely available	[148]
IntApoop	IntApoop can be used to predict potential apoptotic protein interactions, visualize predicted interactions and query global human apoptotic interactions network	[208]
Integrator	Integrator is a web-integrated graphical search tool for protein–protein interaction networks across 50+ genomes	[209]
Inter-Chain Beta-Sheets (ICBS)	ICBS is a database of protein–protein interactions mediated by interchain β-sheet formation	[149]
InterPreTS	InterPreTS predicts interaction through tertiary structure	[210]

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Table 2. Protein–protein interaction analysis software/online servers (cont.).

Software	Description	Ref.
InterViewer	InterViewer is a fast layout algorithm visualizing large-scale protein interaction networks	[211]
IntScore	IntScore is a web tool for confidence scoring of biological interactions, which provides six network topology- and annotation-based confidence scoring methods	[212]
JiffyNet	JiffyNet is a web-based instant protein network modeler for newly sequenced species	[213]
KFC2 Server	KFC2 Server predicts hotspots using support vector machines along with a training set composed of about the same number of hotspot as non-hotspot residues, and incorporating additional features which capture the degree of residue flexibility	[214]
LIBP-Pred	LIBP-Pred is a web server for lipid binding proteins using structural network parameters	[215]
meta-PPISP	A meta server for protein–protein interaction site prediction built on three individual web servers: cons-PPISP, PINUP and Promate	[216]
MoNetFamily	MoNetFamily is a web server to infer homologous modules and module–module interaction networks in vertebrates	[217]
morFeus	morFeus is a web-based program to detect remotely conserved orthologs using symmetrical best hits and orthology network scoring	[218]
Multi-scale Community Finder (MCF)	MCF is a tool to profile network communities (i.e., clusters of nodes) with the control of community sizes	[219]
NASCENT	NASCENT is an automatic protein interaction network generation tool for non-model organisms	[220]
NatalieQ	NatalieQ is a web server for topology-based alignment of a specified query protein–protein interaction network to a selected target network using the Natalie algorithm	[221]
Network Analysis, Visualization and Graphing TORonto (NAViGaTOR)	NAViGaTOR is a software package for visualizing and analyzing protein–protein interaction networks	[222]
NetAlign	NetAlign is a web-based tool for comparison of protein interaction networks	[223]
NetCAD	NetCAD is a web-based tool for systematic investigation of CAD-specific proteins in human protein–protein interaction network	[224]
NetComm	NetComm is a network analysis tool based on communicability	[225]
NetVenn	NetVenn is an interactome network-based web analysis application platform which has an efficient and interactive graphic tool that provides a Venn diagram view for comparing two to four lists in the context of an interactome network	[226]
Network-extracted Ontology (NeXO) Web	The NeXO Web platform is an online database and graphical user interface for visualizing, browsing and performing term enrichment analysis using NeXO and the gene ontology	[227]
NOXclass	NOXclass is a classifier identifying protein–protein interaction types (biological obligate, biological non-obligate and crystal packing) implemented using a support vector machine algorithm	[228]
NRF2-ome	NRF2-ome is an integrated web resource to discover protein interaction and regulatory networks of NRF2	[229]
OpenPPI predictor	OpenPPI predictor is an open source tool for prediction of genome wide protein–protein interaction network based on ortholog information	[230]
Osprey	Osprey is a software platform for visualization of complex interaction networks. Osprey builds data-rich graphical representations from Gene ontology (GO) annotated interaction data maintained by the GRID	[231]
Patch Finder Plus	Patch Finder Plus is an automatic server to extract and display the largest positive electrostatic patch on a protein surface	[232]

Table 2. Protein–protein interaction analysis software/online servers (cont.).

Software	Description	Ref.
PathBLAST	PathBLAST is a network alignment and search tool for comparing protein interaction networks across species to identify protein pathways and complexes that have been conserved by evolution	[233]
PEDANT	PEDANT is a Protein Extraction, Description and ANalysis Tool, which provides exhaustive annotation of nearly 3000 publicly available eukaryotic, eubacterial, archaeal and viral genomes with more than 4.5 million proteins by a broad set of bioinformatics algorithms	[234]
personalized PageRank	personalized PageRank is a program for analyzing protein interaction networks	[235]
PerturbationAnalyzer	PerturbationAnalyzer is a tool for investigating the effects of concentration perturbation on protein interaction networks	[236]
PESCADOR	PESCADOR is a web-based tool to assist text-mining of biointeractions extracted from PubMed queries	[237]
PINAT1.0	PINAT1.0 is a protein interaction network analysis tool	[238]
PINV	PINV is an open source, native web application that facilitates the visualization of protein interactions	[239]
POLAR MAPPER	POLAR MAPPER is a computational tool for integrated visualization of protein interaction networks and mRNA expression data	[240]
PPI spider	PPI spider is a tool for the interpretation of proteomics data in the context of protein–protein interaction networks	[241]
PPLook	PPLook is an automated data mining tool for protein–protein interaction	[242]
PRIMOS	PRIMOS is an integrated database of reassessed protein–protein interactions providing web-based access to *in silico* validation of experimentally derived data	[172]
PRISM2.0	This online tool allows to explore protein interfaces and predict protein–protein interactions	[243]
PRODISTIN	PRODISTIN is a network analysis and protein function prediction	[244]
ProMate	ProMate predicts the location of potential protein–protein binding sites for unbound proteins by evaluating the distributions of protein surface dots	[245]
Protein–Protein Interaction Server	The server provides a means of calculating a series of descriptive parameters for the interface between any two polypeptide chains in a three dimensional protein structure	[246]
Protein Association Analyzer (PRASA)	PRASA is an integrated web server that predicts protein interactions as well as interaction types	[247]
Protein Interactions VisualizatiOn Tool (PIVOT)	PIVOT is a Java-based tool, for visualizing protein–protein interactions. It is rich in features that help the users navigate and interpret the interactions map, as well as graph-theory algorithms for easily connecting remote proteins to the displayed map	[248]
Protinfo PPC	Protinfo PPC predicts the atomic level structure of a protein complex	[249]
Related Structures	The Related Structures tool allows users to find 3D structures from the Molecular Modeling Database (MMDB) that are similar in sequence to a query protein	[250]
Rosetta FlexPepDock	Rosetta FlexPepDock is web server for high resolution modeling of peptide-protein interactions	[251]
SCOWLP	Structural classification of protein binding regions for atomic comparative analysis of protein interactions	[252]
SNAPPI-View	SNAPPI-View predicts interactions between specific domains	[253]
SNOW	SNOW is a web-based tool for the statistical analysis of protein–protein interaction networks	[254]

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Table 2. Protein–protein interaction analysis software/online servers (cont.).

Software	Description	Ref.
SPIDDER	Solvent accessibility based Protein–protein InterfaceiDEntification and Recognition	[255]
SPIDer	SPIDer is a database for a predicted protein–protein interaction dataset which is solely derived from the Gene Ontology (GO), based on the genome of yeast *Saccharomyces cerevisiae*, and provides users with a graphical interface for visualizing an interaction subnetwork for a list of proteins of interest	[162]
Spotlite	Spotlite is a web application with augmented algorithms for predicting co-complexed proteins from affinity purification – mass spectrometry data	[256]
SteinerNet	SteinerNet is a web server for integrating 'omic' data to discover hidden components of response pathways	[257]
STRING	STRING is a database of known and predicted protein interactions. The interactions include direct (physical) and indirect (functional) associations; they are derived from four sources: Genomic Context, High-throughput Experiments (Conserved) Co-expression, Previous Knowledge	[163]
Struct2Net	Struct2Net is a web service to predict protein–protein interactions using a structure-based approach	[258]
SurpriseMe	SurpriseMe is an integrated tool for network community structure characterization using Surprise maximization	[259]
SurvNet	SurvNet is a web server for identifying network-based biomarkers that most correlate with patient survival data	[260]
The Domain Interaction MAp (DIMA)	The DIMA is a web server for exploring the protein domain network	[261]
The Jena Protein–protein Interaction Website	This website gives an extensive introduction about sources for detecting protein–protein interactions	[262]
The Protein Interaction and Molecule Search (PRIMOS) database	The PRIMOS database operates as an integrated knowledge portal for analyzing protein–protein interaction data	[172]
The Proteome Browser (TPB)	This web portal brings together data and information about human proteins from a number of sources and presents them in a gene- and chromosome-centric, interactive format	[263]
TopNet-like Yale Network Analyzer (tYNA)	The tYNA platform for comparative interactomics is a web tool for managing, comparing and mining multiple networks	[264]
Turbine	Turbine is an open-source network analysis package for simulating network dynamics, particularly for experiments involving perturbations	[265]
Vector Alignment Search Tool (VAST)	A computer algorithm that identifies similar protein 3D structure, which is able to identify distant homologs that cannot be recognized by sequence comparison alone	[266]
Integrative Visual Analysis Tool for Biological Networks and Pathways (VisANT)	VisANT is an integrative platform for network/pathway analysis	[267]
webPIPSA	webPIPSA is a web server for the comparison of protein interaction properties	[268]
WHISCY	WHISCY is an online scoring system evaluating protein–protein interaction propensity based on the conservation of functional motif sequence	[269]

more closely reflect the native environment of the proteins under study. Three major methods have been used: the Y2H system, PCA and MAPPIT (Figure 1). These methods are based on a common principle of 'molecular fishing' and typically require two key components: bait protein and prey protein [18].

The yeast two-hybrid system, which was first described by Fields and Song in 1989 [30], has been extensively used to investigate PPIs. Y2H is based on the complementation of two halves of a transcription factor bound to the two proteins whose interaction is to be tested (Figure 1) [31]. Importantly, data obtained by the Y2H method are not significantly biased toward any particular functional characterization [32]. The classic Y2H system consists of two special function domains: the DNA-binding domain of the transcription factor, which is bound to the molecule of interest (namely the bait) and the activation domain of the transcription factor, which is bound to the potential interaction partner (the prey). If the bait and prey proteins interact with each other when expressed in a yeast cell, an intact and functionally active transcription factor is formed from the binding domain and activation domain, and transcription of a reporter gene (usually β-galactosidase activity) is activated [33]. The main advantages of this method include speed and simplicity, low cost and a broad application ranging from the exploration of PPIs to large-scale interaction mapping [34,35]. Nevertheless, there are also several limitations with the Y2H system. Y2H screens have been criticized for generating high rates of false-positives [36]. The exact false-positive rate may be as high as 50% of the identified interactions [37]. Additionally, forced subcellular localization of bait and prey proteins in the yeast nucleus may prohibit certain interactions, including interactions involving integral membrane proteins, which are major therapeutic targets [38]. Likewise, proteins which require post-translational modifications (e.g., interactions between phosphorylated proteins and SH2-domain-containing proteins [39]) for functionality are unlikely to behave or interact normally under Y2H conditions [40]. Several alternative technologies based on the concept of functional reconstitution of a transcription factor have been developed to deal with the limitations of the original method, including Nuclear Two-Hybrid Systems, Membrane-Localized and Secretory Pathway Two-Hybrid Systems [33].

PCAs are also *in vivo* methods of PPI detection. They utilize the direct physical interaction of two proteins to reconstitute a reporter protein from two genetically attached non-functional fragments (Figure 1). After reconstitution, the protein can then activate a reporter, or act as a reporter *per se*. In contrast to Y2H systems, PCAs depend on PPI-induced refolding of two protein fragments to reconstitute a functional reporter and usually do not require specific localization, therefore, more closely reflecting the native environment of the proteins under study [33]. Importantly, PCAs do not require subsequent transcription or other amplification [41]. An obvious advantage of PCAs over two-hybrid systems is that they can be applied to specific organisms of interest. A number of reporters have been used for PCA, including split murine dihydrofolate reductase [42] and yellow fluorescent protein [43]. It should be noted that PCAs are also known as 'split-protein sensors' [33], and specific PCA techniques are named split-'X' methods (e.g., split-DHFR and split-FP).

MAPPIT, which is based on reconstitution of a true mammalian signal transduction pathway (the Janus kinase-signal transducer and activator of transcription cascade) that is initiated by interaction between the bait and prey, is another *in vivo* method for detection of PPIs. After the receptor (prey) has been stimulated with ligand (bait), their interaction drives activation of a signaling cascade resulting in STAT3-dependent reporter gene activation (Figure 1) [44]. A clear advantage of MAPPIT is that it operates in intact mammalian (human) cells [45]. Proteins that require post-translational modifications to carry out their function can behave or interact normally with their partners, and the sensitivity and robustness of MAPPIT can be better than the Y2H system. However, the scalability of MAPPIT is much lower than Y2H, because the complexity of handling and the cost of working with mammalian cells is much higher than with yeast [46]. Also, the interaction sensor of MAPPIT is anchored to the plasma membrane and may preclude the analysis of PPIs at their native subcellular localization. Thus, KInase Substrate Sensor, a new derivative MAPPIT approach, has been recently developed to overcome this limitation [47]. In contrast to MAPPIT, in KInase Substrate Sensor the bait and prey proteins can be either soluble or transmembrane proteins, enabling analysis of PPIs between or among both protein classes (Figure 1) [47].

In vitro *methods for the detection of PPIs*

With *in vitro* methods, the procedures are carried out in a controlled environment outside the living organism. Methods include TAP-MS, protein microarrays and the LUMIER method.

Affinity purification is an important method for isolating protein complexes. In AP-MS, the bait protein is armed with an affinity tag, allowing identification of captured protein complexes by MS following pull down [48,49]. Many types of ligands can be used in this approach including oligonucleotides [50], chemical moieties [51] and proteins [52]. It is possible to carry out direct molecular fishing and affinity purification on optical chips and paramagnetic nanoparticles including surface plasmon resonance (SPR) validation for proteomics analysis [53,54]. Also, with the combination of newly developed MS technologies (e.g., surface plasmon resonance imaging in arrays coupled with MS, biomolecular interaction analysis-MS) and some optical SPR-biosensors (e.g., BIAcore 3000, Iasys, Horiba), biosensor-based micro-affinity purification can provide an excellent platform for proteomics analysis that can be readily used in the field of affinity-MS [55–58].

To overcome high false-positive rates, a derivative form of AP-MS has been developed that eliminates this disadvantage: TAP [59]. In TAP, the tag fused to the target protein is composed of two sequential affinity tags, protein A having very high affinity for IgG and a calmodulin binding peptide having

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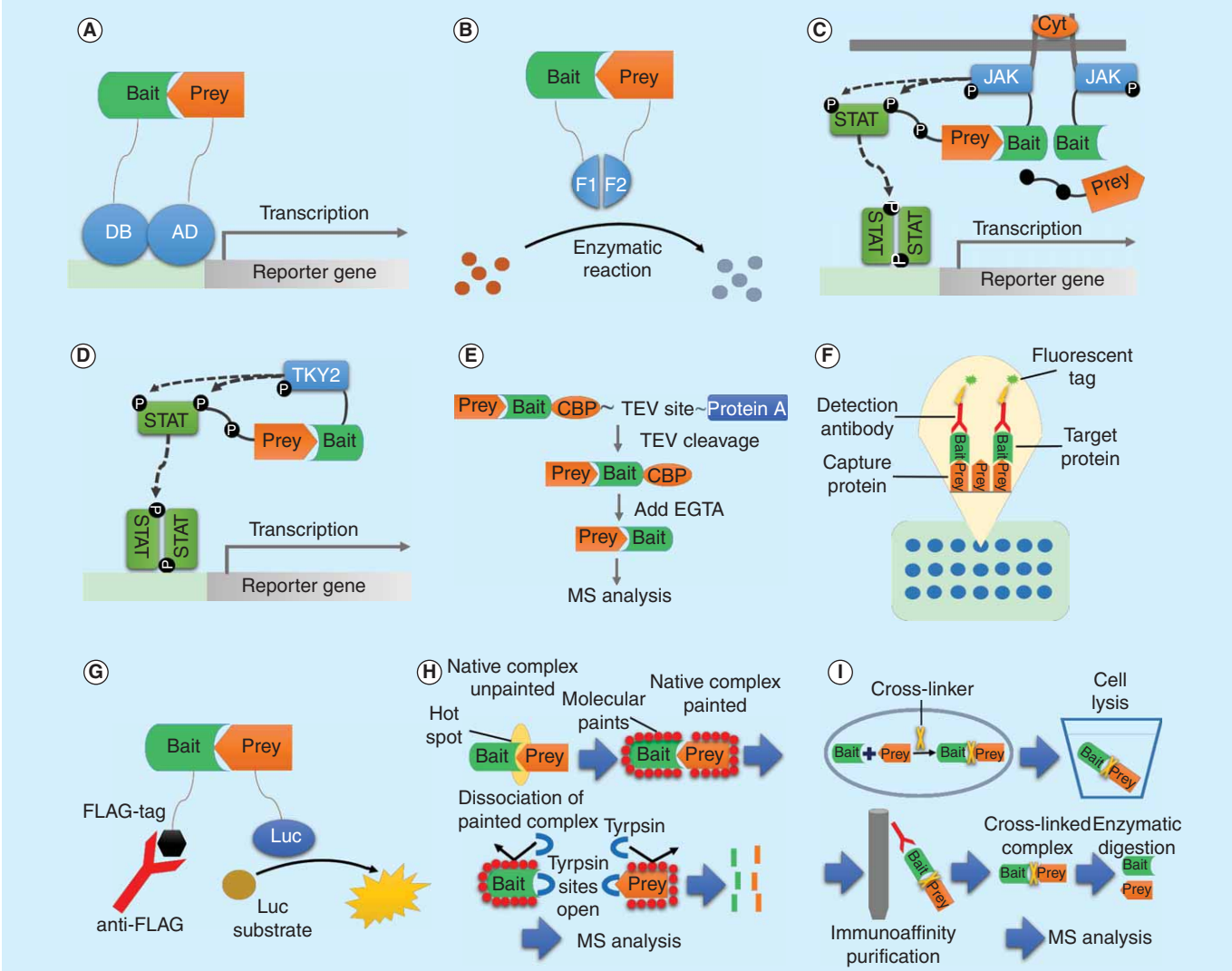

Figure 1. Methods for the detection of protein–protein interactions. (A) Y2H: bait and prey are fused to the DB and AD of a yeast transcription factor, respectively, and co-expressed in yeast cells. The interaction of bait and prey will form an intact transcription actor and further perform transcription of the reporter gene. **(B)** PCA: the bait and prey proteins are fused to non-functional halves of a protein. Upon interaction of the bait and prey, the reconstitution of reporter protein will perform enzymatic activity and transform the substrate (brown circles) to a detectable end product (gray circles). **(C)** MAPPIT: bait proteins are coupled to a signaling-deficient cytokine receptor (gray cylinders). Prey proteins are tethered to another receptor-moiety harboring STAT-recruitment sites (gray line). Upon bait–prey interaction, a functional receptor is reconstituted and can be activated by a cytokine ligand. This ligand-binding leads to cross-phosphorylation of JAKs, which in turn phosphorylate the prey receptor-fragment, rendering the receptor accessible to STAT docking (P represents the phosphorylated sites). Recruited STATs are subsequently phosphorylated by JAKs, then dimerize and enter the nucleus to activate reporter gene expression. **(D)** KISS: bait is fused to a kinase-containing portion of TYK2 (tyrosine kinase 2) and prey is coupled to a gp130 cytokine receptor fragment. When bait and prey interact, TYK2 phosphorylates STAT3 docking sites on the prey chimera, which ultimately leads to activation of a reporter gene. **(E)** TAP-MS: a two-step purification is used: the first uses Protein A, which binds to IgG beads. The Protein A is subsequently removed by TEV protease. The second purification uses the affinity of a CBP for calmodulin, which is removed by EGTA. And the interaction protein complexes are further identified by MS. **(F)** Protein microarrays: prey proteins are immobilized on the array surface and incubated with bait; detection is carried out by means of a labeled secondary antibody. **(G)** LUMIER: the bait is fused to Renilla luciferase (Luc), the prey is FLAG-tagged (black), and co-expressed in mammalian cells. Cell lysates are treated with anti-FLAG antibodies for immunoprecipitation. Subsequent light emission upon addition of the luciferase substrate correlates with a bait–prey interaction in the pull down. **(H)** Protein painting: paint molecules coat the surface of native protein complexes but cannot gain access to solvent-inaccessible protein–protein interface regions. Trypsin cleavage is blocked by the presence of paint molecules that bind non-covalently near trypsin consensus sequences. Following dissociation of painted proteins, the area of interaction remains unpainted and is susceptible to trypsin cleavage. **(I)** Chemical crosslinker: live cells expressing the epitope-tagged protein of interest are treated with chemical crosslinker. The crosslinking reaction is quenched and the cells are lysed. Proteins crosslinked to the tagged protein of interest are purified by immunoaffinity, followed by enzymatic digestion and LC/MS/MS analysis.
KISS: Kinase substrate sensor; MAPPIT: Mammalian protein–protein-interaction trap; MS: Mass spectrometry ; PCA: Protein-fragment complementation assay; TYK2: Tyrosine kinase 2; Y2H: Yeast two-hybrid.

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a high affinity for calmodulin: these are separated by a cleavage site of tobacco etch virus protease (Figure 1) [60]. Two rounds of purifications are required in this approach [61]. During the first purification step, the TAP-tagged target protein is purified using a protein A affinity support followed by incubation with tobacco etch virus protease which releases the target protein. In the second affinity step, the protein complex is further purified via the calmodulin binding peptide of the TAP tag in the presence of calcium and released by use of calcium-chelating agents (usually ethylene glycol tetraacetic acid) [36]. Finally, the purified protein complexes are identified and characterized by MS. The TAP-MS strategy has several advantages over other methods. MS provides sensitive and specific protein identification and the method has the ability to examine interactions between multiple proteins [31]. TAP-MS is a generic method because the corresponding cDNA of proteins can be readily cloned. The two rounds of purification enhance the specificity and sensitivity of this method [61]. However, despite these advantages, the TAP-MS method also has limitations. One potential problem is that the increased purity and specificity come at increased cost, and transient affinity PPIs may be lost during the series of purification steps [62]. Additionally, identification of low-abundance binding partners requires a relatively large amount of starting material for success [63]. Apart from TAP tag, there are many other epitope tags that are applicable for AP-MS, either alone or in combination. Also, a number of purification options are available, each with their own strengths and weaknesses. A discussion of this and suitable choice of AP-MS strategy can be found in recently published reviews [64,65].

To ensure high-quality results, raw data must be processed to exclude contamination, false positives and background effects. To achieve this, both filtering against contaminant lists and scoring using methods such as SEQUEST, MASCOT, X!Tandem MiST, SAInt and CompPASS, are powerful options [64,65]. Since traditional methods are limited when probing subcellular localized interactions and non-soluble protein complexes, novel tactics are required. To this end, an improved version of AP-MS, called multiple cell compartment AP-MS/MS (MCC-AP-MS/MS), has been developed, which enables researchers to independently identify PPIs in either cytoplasm, nucleoplasm or chromosomes with better sensitivity [66].

Protein microarrays have emerged as a promising and powerful means of detecting PPIs as they are rapid and efficient, allowing high-throughput detection. A protein microarray comprises a suitable surface on which functional target proteins have been affixed at defined separate locations in an ordered manner allowing subsequent functional interrogation using enzymatic or binding assays (Figure 1) [67,68]. Surfaces such as glass slides, polyacrylamide gel pads, nitrocellulose membranes, gold, silicon, lipid bilayers or microwells have been used for protein printing [69,70], although each has its own advantages and drawbacks. Two general strategies have been used to generate the proteins for microarrays. In the classical approach, proteins to be arrayed are produced by heterologous expression in *Escherichia coli* and then purified and spotted onto the array surface [71]. To avoid the need to both express and purify proteins for arrays, a more recent *in situ* synthesis method has been developed to generate protein microarrays by direct protein synthesis on a suitable surface [72].

Protein detection techniques for microarrays can be classified as either label-based or label-free methods. Historically, label-based protein microarray have been most widely used, with most applications using fluorescent dyes [73], chemiluminescence or radioactive labeling for detection [74]. More recently, techniques like quantum dots, gold nanoparticles, dye-doped nanoparticles and bead-based methods have also been developed [75]. However, label-based detection strategies have their limitations including synthetic challenges, the time and effort required for labeling, interference due to the presence of the tag and multiplexing issues [75]. Therefore, label-free techniques have been developed to measure intrinsic properties of the target spot (e.g., changes in mass or dielectric properties at the sensor surface). Techniques including SPR/SPRi, nanohole arrays, carbon nanotubes, microcantilevers, nanowires are at various stages of development [75,76]. Importantly, label-free methods often provide quantitative information for the binding kinetics since the concentration of both bait and prey can be controlled. Competition-type assays can also be established [77]. However, instrument costs and sample spotting anomalies can limit their uptake [76]. In general, protein microarrays offer a high-throughput approach to interrogate potential binding partners and obtain quantitative information on binding affinities [78].

LUMIER, a method that combines two-hybrid and biochemical strategies, was developed for high-throughput PPI analysis in cultured mammalian cells [79]. In this strategy, bait proteins are genetically fused to an affinity tag (usually FLAG) for purification, and prey proteins are attached to Renilla luciferase enzyme for detection (Figure 1). The affinity tag insures purification of the protein complex of interest, and Renilla luciferase enzyme subsequently reports the presence of the interaction partner. LUMIER was originally applied to map the TGF-β pathway network, where key proteins were fused to Renilla luciferase, co-immunoprecipitated with 518 FLAG-tagged library prey proteins and subsequently assayed for luciferase activity [79]. However, it should be noted that although overexpressing of proteins in LUMIER may be beneficial in assaying weak or transient interactions, it may also yield some false-positive results [80].

Identification of PPI hotspots

Direct detection of PPIs as described above is helpful in deciphering the underlying mechanisms of biological processes. However, deeper understanding of biomolecular interactions will enable deeper mining of PPIs and even the design of drugs targeting disease-relevant interactions. To this end, precise location of interacting peptides or residues, in other words, the hotspots, is required. Here, we introduce two powerful methods addressing such a challenge.

Protein painting is a recently developed method for detecting drug targets hidden within native protein–protein interfaces.

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The rationale is to paint the outside of a biological complex with proteolytic-resistant small molecule paints that can tightly coat the interacting surfaces (Figure 1). After painting a complex with such paints, only the uncoated interacting protein–protein interface can be digested by trypsin, which generates peptides specifically responsible for a PPI and analyzable by MS [81]. With this method, our understanding of PPI will be further promoted as the properties of PPI interfaces are to be more precisely unveiled.

Chemical crosslinking is another approach for detecting specific interacting peptides or residues. A crosslinker with defined length is connected via covalent bonds to functional groups of amino acid side chains, allowing the crosslinked amino acids to be identified by MS (Figure 1). A range of chemical crosslinkers, including photo-initiated crosslinkers, have been widely applied to identify PPIs [82]. Most chemical crosslinking studies have been performed in a 'bottom-up' fashion, where, after the crosslinking reaction, the covalently connected proteins are digested enzymatically and the resulting peptides are analyzed by liquid chromatography tandem MS [83–85]. More importantly, recent improvements have made it possible to define PPIs in living cells, mainly due to the advent of a novel cell membrane permeable chemical crosslinker [86]. Due to its convenience and powerfulness, it is one important tool for identification of hot spots.

Analysis of interactome data

Validation

Although both the traditional and more recently developed high-throughput methods have contributed significantly to the large-scale profiling of the interactome, there are still roadblocks to its progress. Lack of confidence in the data and limited coverage are two major barriers for its use in addressing scientific questions [10]. It has been reported that more than half of the reported protein interactions in the early yeast proteome were false positives while only about 30% could be redetected by orthogonal methods [10,87]. It appears that nonspecific interactions, impurity of separated complexes resulting from contamination by abundant proteins and intrinsic defects of bait proteins were the major causes for the high false-positive rate. Low coverage seems to be due to a number of reasons including lack of post-translational modifications in the heterogeneously expressed proteins, lack of sensitivity for low abundance proteins, instability of weak or transient interactions, problems with membrane protein interactions, mechanistic limits of testing techniques, the coverage of detectable interactions in a single mapping round, spatial-temporal effects and stimuli specificity.

Clearly, validation using orthogonal methods is essential to guarantee the reliability of the data, especially when the research objective is to develop disease markers, or to identify disease-relevant PPIs. Commonly used methods include PCA, MAPPIT and LUMIER [41]. Although all these techniques are suitable for validation, there remain caveats. As the sensitivity of each technique differs, data overlap may not be apparent. It has been reported that only 20–35% of data generated by different techniques, including Y2H, PCA, LUMIER and MAPPIT, are comparable [10,46].

Benchmarked methods play a vital role in addressing false positives, as differences in selection of the reference list lead to distinct outputs. Two reference sets, a positive reference set (PRS) and a negative reference set (NRS), are required. The content of the reference set has an enormous impact on reliability, as any bias will lead to errors. In principle, data that have been verified in at least two independent studies are suitable as a PRS. To deduce the bias, one recommended PRS is the use of a single complex (e.g., the ribosome) as the interactions are not spatially-temporally constrained. Selection of the same class of data is also important [41,88]. The size of a reference set also plays a key role. Usually, larger reference lists give better accuracy, with ≈1000 pairs less biased with increased statistical power. In addition to the absolute size, the appropriate relative size of PRS and NRS to test data is important and will depend on the specific applications [41].

Network structure & topology

As interactomes are complex networks, approaches used for common network analysis are also suitable for data interpretation. Two fundamental properties of a network are degree of distribution and the clustering coefficient. The former represents the average number of protein interactions of a certain protein within a certain network. The later describes the connectivity density between the protein's interactors. Usually, a high clustering coefficient is associated with a highly connected sub-network [89]. A major focus has been to identify hubs, which are proteins highly connected with other proteins, since such connections are frequently physiologically relevant (Figure 2).

Taylor *et al.* [90] devised a novel way to analyze new features of hubs based on the observation that, due to spatial and temporal effects, some protein interactions happen conditionally within a cell while some interactions happen constantly regardless of it, and applied this to breast cancer prognosis. They obtained genome-wide expression data from 79 human tissues, and analyzed the extent to which a hub and its interacting partners were co-expressed in the same tissues. Then, after measuring the average Pearson correlation coefficient of selected hub proteins, the authors classified the hubs into context-specific ones (called intermodular hubs) where their interacting proteins are not always co-expressed, and constitutive ones (called intramodular hubs) where their interacting partners are always co-expressed (Figure 2). After analyzing the network features of the two kinds of hubs (including betweenness and characteristic path length [defined in the article]), they found that intermodular hubs tend to spatially and temporally provide links to intramodular hubs, while intramodular hubs often fulfill specific functions as key interacting modules in a sub-network that is typically represented by a macromolecular complex. These specific properties suggest functional differences between the two kinds of hubs. Consistently, intermodular hubs were found to be larger and have more domains, including a number of

cell signaling domains (e.g., tyrosine kinase, PDZ and Gα domains), while intramodular hubs were smaller, had larger domains with signaling only rarely involved. Moreover, intermodular hubs tend to comprise more post-translational modifications and short binding motifs, suggesting that intermodular hubs may play multiple roles and are highly dynamic while intramodular hubs tend to be more conserved and fundamental. Since intermodular hubs are vital to signal flow to intramodular hubs, and intramodular hubs control effectors under some circumstances, it is likely that disturbance of signal transduction between these two kinds of hubs due to either intermodular hub mutation or intramodular hub dysfunction would occur in a cell under diseased conditions. To address this hypothesis, 256 hubs from poor-outcome breast cancer patients were compared with those from surviving patients [90], BRCA1, a tumor suppressor that is mutated in a subset of familial breast cancers was a significantly altered intermodular hub protein. There was strong correlation with the expression of its partners in tumors from surviving patients, but not with their expression in tumors from poor-outcome patients [90–92]. Similarly, although the expression of the oncogenic protein SRC was not significantly different between the two patient groups, connection with its interacting partners was clearly affected. Taken together, this analysis of interactome network structure led to the identification of two different kinds of hubs in the human interactome, which were found to play distinct roles in signal transduction and impact on our understanding of cell signaling transduction processes.

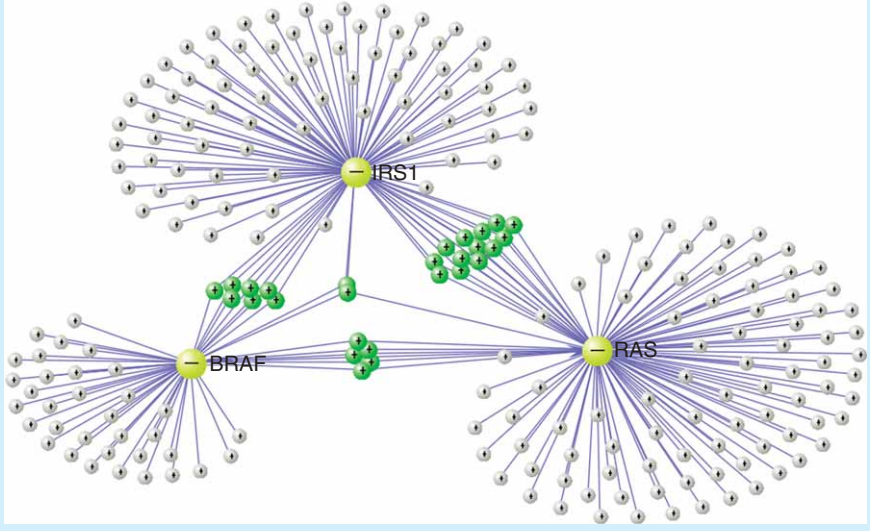

Figure 2. Schematic showing interactome topology. Proteins are classified into three types in this figure according to their different degrees of distribution. Light gray circles connect with only one partner, the green circles have two or more than two interactors and the yellow circles represent those with numerous interacting partners. In addition, BRAF and IRS1 (insulin receptor adaptor protein) are intermodular hubs while RAS is an intramodular hub in this network. This schematic is produced with VisAnt, an online java tool.

The model of classifying hubs into intermodular and intramodular types is further supported by other studies, although more complex network structures are indicated [93]. Apart from classification of hubs into intermodular hubs and intramodular hubs, there are numerous other knowledge-based methods for the analysis of proteomics data, which is presented in a recent review [89]. To conduct these or similar PPI network analyses, further information and protocols can be found in a recently published article [64].

Comparative interactomics

Comparative interactomics is based on the concept that molecular mechanisms underlying fundamental biological processes are conserved across species. The divergence of protein interaction profiles among closely related organisms is measured allowing the correlation of specific traits to phenotypic differences and providing information about the evolutionary mechanisms leading to organism diversity [94]. Several methods have been developed in addition to directly comparing known orthologous proteins to find correlated protein interactions in evolutionarily distant species, for example, to study protein conservation and variation at the amino acid sequence level [95,96]. It is believed that pairs of residues that are part of two interacting surfaces tend to co-evolve. Thus, direct analysis of the sequences of interacting proteins provides direct evidence for protein interaction pair co-evolution. Apart from detecting amino acid sequences, discerning matches of functional motifs in different species can also be used [96]. Several tools such as Automated Detection and Validation of Interaction by Co-Evolution and Conserved Domain Database are available for this kind of study (Tables 1 & 2).

However, some doubts on reliability have been raised as the overlap between species has been found to be less than expected [96]. For example, a study comparing the interactomes of human and *Caenorhabditis elegans* found that even with interactome sizes of >25,000 and 5000 interactions for human and *C. elegans*, respectively, <300 interactions could be compared [97]. Interactions and network structure are shaped by selective pressure. Physiological constraints could enforce network stability or, by contrast, promote the rapid emergence of alternative advantageous protein connections leading to network divergence. This partially depends on interactome redundancy, the level of which is relatively unknown [94]. The yeast and human interactomes evolve at rates of 100 or 1000 interactions changed (gained or lost) per million years in recently duplicated genes [98]. Every 300 million years, as many as half of all interactions in a yeast interactome may be replaced by new interactions [99].

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Application of the interactome

Understanding of cell/disease biology

Uncovering PPIs involved in key cell regulatory systems is fundamental to understanding cell biology and has obvious implications for health and disease. Zafar *et al.* successfully unveiled a novel regulator, pyruvate kinase isozyme M1/M2 (PKM2), of Δ23–230 cellular prion protein (PrP^C) in the prion protein-deficient murine hippocampus (HpL3-4) neuronal cell line by applying chromatography/MS analysis to truncated/anchorless Δ23–230 PrP^C along with its interacting proteins [100]. As PrP^C is associated with Gerstmann–Sträussler–Scheinker neurodegenerative disease, this discovery may help understand the disease mechanism.

Functional proteomics/interactomics has also been used to reveal dynamic changes in signaling networks related to cancer. In particular, the tyrosine kinase signaling pathways have been addressed. A recent excellent review by Kolch and Pitt has addressed this topic, focusing on the EGFR, breakpoint cluster region-ABL1 and ERK signaling pathways [101].

Interactomics was successfully used to map the TNF-α/NF-κB signaling pathway that is involved in numerous physiological and pathological processes. Using a multidimensional approach combining tandem affinity purification, liquid-chromatography tandem-MS/MS, bioinformatics, network analysis and molecular biology (directed functional perturbation studies using RNA interference), 221 molecular associations and 80 novel interacting partners were identified, of which 10 were new functional modulators [102,103].

More recently, researchers have used chemical crosslinking and MS to determine both PPIs and residue proximities *in cellulo*. Crosslinking between lysine residues with a 12 Å MS labile chemical crosslinker was used to obtain residue-specific interaction data for the CRISPR RNA-silencing complex. This supported the cryo-EM map of the complex they had generated and clearly identified Cmr4 as the conserved endoribonuclease of the Cmr complex [85]. Subbotin and Chait have recently used crosslinking and MS as part of a pipeline to probe transient interactions in the nuclear pore complex. Their protocol involved flash-freezing cells in liquid nitrogen to preserve the cellular environment at the moment of freezing; cryomilling to fracture the frozen cells into intact sub-micron chunks to allow for rapid access of chemical reagents and to stabilize the intact endogenous subcellular assemblies and interactors upon thawing and using the high reactivity of glutaraldehyde to achieve sufficiently rapid stabilization at low temperatures to preserve native cellular interactions. They termed this method Stabilized Affinity Capture-MS [104].

BRCA1 has been extensively studied because of its role in genome integrity and its association with cancer susceptibility [105,106] and systematic mapping of its interactome has been conducted [107]. Recently, a bipartite screening using both Y2H and TAP-MS methods revealed a number of new interactors of BRCA1. These include TONSL, SETX, TCEANC and TCEA2, which all participate in maintaining genome integrity. Further experiments exploring the function of such interactions revealed that BRCA1 helps reboot transcription after UV damage and aids in the prevention or repairing of DNA damage caused by stabilized R loops [108].

Uncovering disease-causative PPIs is an alternative method for developing novel treatments, and with the development of interactome profiling techniques, this strategy has shown potential. A recent study was conducted to identify lamin A interacting proteins responsible for alternative pathologies. Mutations in the lamin A gene cause laminopathies, a collection of phenotypically diverse diseases including muscular dystrophies, cardiomyopathies, lipodystrophies and premature aging syndromes. Performing a Y2H screening using the human ORFeome V5.1 library, numerous interactions between lamin A and its interactors were detected. Three hundred and thirty-seven lamin A binding proteins were identified and validated. Fifty disease-relevant interactions were also uncovered by testing the identified interactors with 89 known lamin A disease mutations. A focus on the interactions between progerin, the lamin A isoform responsible for the premature aging disorder Hutchinson–Gilford progeria syndrome, and its interactors found that the interactors were mediated by farnesylation. The IgG-like domain was identified as an interaction hotspot by mapping the interaction sites on lamin A [109].

Biomarker discovery

Biomarkers play an important role in medicine for early diagnosis, staging, surveillance, prognosis and treatment selection. Interactomics approaches to biomarker discovery have the advantage that they provide a functional context in which to analyze the mechanistic role of genes or proteins found to be differentially expressed by proteomics analysis. To identify protein 'signatures' that were discriminative of late-stage colorectal cancer, Nibbe *et al.* [110] used a two gel-based proteomics approach to identify significantly differentially expressed proteins between non-diseased and late-stage tumor tissues obtained from 12 patients. Sixty-seven proteins were identified which were used to generate protein–protein interaction subnetworks revealing potential mechanisms of tumorigenesis specific to that phenotype. Similar approaches have been used to find potential biomarkers for breast cancer, lung cancer (using a phosphopeptide enrichment strategy) and Ewings sarcoma (using affinity purification of the nucleophosmin complex) [111–113]. In a recent study, to detect cervical cancer biomarker patterns in blood plasma and urine, Garbett *et al.* initially identified proteins that significantly changed calorimetric characteristics in patients with cervical cancer using differential scanning calorimetry. They hypothesized that this reflected differential expression of disease biomarkers that subsequently bound to and affected the thermal behavior of the most abundant plasma proteins. The interactome of these thermally modified proteins were then generated and a list of cervical cancer biomarkers was revealed [114].

One advantage of the analysis of disease-related interactomes is they reveal networks of multiple genes and proteins which may act as biomarker panels [115]. Chuang *et al.* compared the interactomes of metastatic and non-metastatic breast cancer

patients and identified a subset of the interactome indicative of metastatic breast cancer. They found that the subnetwork markers were more reproducible than individual marker genes selected without network information, with higher accuracy for the classification of metastatic versus non-metastatic tumors. Additionally, the subnetwork suggests the molecular basis for the disease as the components of the network are associated with tumor progression and allude to the possibility that some causative disease gene mutations function through interconnecting many differentially expressed genes [10,116]. It has been suggested that in an evolutionary context, these modules (or subnetworks) are selected for the growth advantage they confer in cancer [115].

Another approach for discovering new biomarkers is to test the differences in relative expression levels of hubs with each of their interacting partners. By selecting protein pairs with highly altered co-expression level as biomarkers, it may be possible to predict cancer patient prognosis [90]. This approach has been shown to be effective when compared with existing prognosis prediction methods [10,90]. Another study that further supports the importance of hub proteins for understanding disease progression and identifying biomarkers was conducted by Li *et al.* Three highly connected protein interaction networks were generated corresponding to early phase, middle phase, and late phase of human prostate cancer by analysis of integrated protein interaction-gene expression networks. After a selection process for the overlapped modules, 94 candidate disease-relevant genes were chosen. Functional analysis using GenGo showed that they were mostly enriched in the nucleus and acted as transcription factors. Further comparison with public databases indicated that these genes are largely disease relevant and many of them were hubs in the interactome. Candidate module biomarkers were validated statistically by their predictive ability to predict prostate cancer progression [117].

Drug target discovery

PPIs are the backbone of cellular function, which makes them attractive therapeutic targets for small molecule drugs [118]. Small molecules targeting PPIs are more convenient to use and importantly can typically be administrated orally without degradation or deactivation in the gastrointestinal tract [118]. The number of human PPIs has been estimated to be between 130,000 and 650,000 [119,120], many of which will be disease related, affording a large drug target pool.

Applying interactomics to disease-relevant PPI discovery is an emerging trend. As discussed above, a typical interactome contains both intermodular hubs and intramodular hubs, with the signal flow between them critical to cell fate [90]. Thus, focusing on the interactions between intermodular and intramodular hubs would appear to be a feasible approach for discovering potential disease-relevant PPIs. In addition, chemical proteomics strategies to assist in the understanding of drug interactomes can promote the optimization of lead compounds and improve drug selectivity and specificity, leading to reduced side effects [103].

In the former section, we have shown that interrupted signal transduction between intermodular and intramodular hubs are disease causative, as exemplified by BRAC1 and breast cancer [90,92,121]. BRAF and RAS were identified as intermodular and intramodular hub, respectively, and an interaction between them had been identified [90], which suggested an important role of it in regulation of biological processes. In accordance, a separate study pointed out that, under normal physiological conditions, the two hubs only interact transiently and carefully control, through the RAS-ERK-MAPK signaling pathway, a number of cell processes, including survival, proliferation, senescence and differentiation. However, in some diseased conditions, including melanoma, the interaction becomes constitutive, resulting in continuous activation of the RAS-ERK-MAPK signaling pathway, which facilitates cancer cell proliferation and survival [122]. Taken together, these studies indicate the power and potential of an interactome in discovering targetable disease-relevant PPIs, which will assist the development of new drugs.

An emerging area where interactomes can facilitate drug target discovery is the pathogen–host interaction. Drugs to specifically target pathogens are limited in number due to the limited size of pathogen proteomes. Additionally, current drugs rapidly loose effectiveness due to highly active drug-resistant mutations of pathogen genes. Mapping of the interactomes between humans and pathogens and targeting host–pathogen PPIs is opening new horizons. Importantly, this strategy offers significant advantages compared with traditional protein antagonists, because new drugs directly target with the host proteins that are much less likely to be mutated. Such drugs may be less toxic as they target specifically disease-related PPIs rather than substrates that are required for normal cellular function [118].

Integrating interactome with other kinds of data, such as gene expression levels, protein–DNA interaction study and phenotypic data, is another strategy to explore novel drug target. This approach has been reviewed in depth recently [10,118].

Expert commentary

Although many novel techniques have been developed for mapping interactomes and the field has moved forward significantly over the last 10 years, a number of challenges still remain. It is difficult to find a generic technique to measure all proteins simultaneously as protein sizes range from several amino acids to many thousands. Additionally, the concentration of individual proteins within a tissue ranges over many logs, complicating the detection of low abundance proteins, some of which will fall outside the current limits of detection. Coupled with this, the dynamic nature and range of affinities further complicates the picture. The subcellular localization of proteins may also hinder the detection of PPIs, particularly for membrane–protein interactions, although improved methods for membrane proteomics are being developed [123]. Moreover, many proteins undergo post-translational modifications that modulate their function, but are not always easy to analyze. While continual improvements are being made in the requisite technologies,

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currently it is necessary to use orthogonal techniques to both populate and validate the human interactome.

Numerous bioinformatic analysis algorithms have been developed (Tables 1 & 2) to mine the interactomes and have contributed significantly to addressing many key biological questions and opening new horizons in medicine. However, interactomics datasets are becoming increasingly large and multi-dimensional placing additional demands on the existing methodology. It is important to combine bioinformatic analysis with biological studies [90]. The combination of interactomics with other 'omics' strategies (e.g., genomics, transcriptomics) will prove synergistic [124].

In the area of drug discovery, the development of small molecule inhibitors of PPIs has shown potential, opening up the way for the development of novel drugs with reduced side effects and drug resistance. Advances have also been made in the use of interactomics to discover novel biomarkers and biomarker panels, although to date these remain to be fully validated on large clinical cohorts and approved for clinical use.

Five-year view

While rapid advances have been made in the field of interactomics since the introduction of the Y2H technology in detecting binary PPIs [125], the field is still constrained, to some extent, by both accuracy (false positives) and coverage. While at present it is prudent to use orthogonal techniques to validate PPIs, as has so often been the case, advances in technology will be key to progress, with individual techniques producing more reliable and complete biomolecule interacting networks. The size and complexity of the interactome means high-throughput PPI mapping techniques will be essential. Techniques such as affinity purification combined with sequential window acquisition of all theoretical spectra (AP-SWATH) MS to study the dynamics of a protein network will promote our understanding of the functional changes in components of a biological complex [126], while top down proteomics will assist the analysis of post-translational modifications [127,128]. Importantly, the cross-linking techniques which have recently been reported [81,129] will facilitate analysis of weak, transient or rapidly exchanging interactions, and help differentiate proximal interactions from those that are more distal [85]. Multidisciplinary approaches such as combining bioinformatics with structural biology will prove synergistic in promoting advances in the field which will benefit the development of personalized medicine.

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Key issues

- As evidenced by the large number of recent publications, interactomics has become a dynamic and rapidly growing field of research with new paradigms in signaling emerging. Currently, over 170,000 human PPIs are registered in the Interologous Interaction Database (Table 1).
- A number of technologies have been adapted for detection of PPIs, including yeast two-hybrid, tandem affinity purification-mass spectrometry, the protein-fragment complementation assay, the luminescence-based mammalian interactome, protein microarrays and the mammalian protein–protein interaction trap.
- These methods can be roughly divided into three groups: *in silico*, *in vivo* and *in vitro*. Each group has both advantages and disadvantages.
- Similar techniques can be used to address other interacting biomolecular interactions including DNA, ligands for enzymes, transporters, receptors, metabolic networks, *etc.* It can also be applied to other systems (e.g., plants).
- Validation of experimentally acquired data is still a demanding procedure, as reliability, high false-positive rates, low coverage and difficulties in multiplexed quantification can hinder the construction of a reliable interactome. Orthogonal methods are required for extended coverage and validation.
- The focus on the differences between intermodular and intramodular hubs is an important new approach for analyzing an interactome.
- Protein interactome maps are proving extremely valuable for deciphering cell/disease biology and discovering new drug targets.
- Identifying subsets of disease-related proteins from interactomes should lead to novel biomarkers and biomarker panels.

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