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Review

High-throughput and multiplexed protein array technology: protein–DNA and protein–protein interactions

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Abstract

Miniaturized protein arrays address protein interactions with various types of molecules in a high-throughput and multiplexed fashion. This review focuses on achievements in the analysis of protein–DNA and protein–protein interactions. The technological feasibility of protein arrays depends on the different factors that enable the arrayed proteins to recognize molecular partners and on the specificity of the interactions involved. Proteome-scale studies of molecular interactions require high-throughput approaches for both the production and purification of functionally active proteins. Various solutions have been proposed to avoid non-specific protein interactions on array supports and to monitor low-abundance molecules. The data accumulated indicate that this emerging technology is perfectly suited to resolve networks of protein interactions involved in complex physiological and pathological phenomena in different organisms and to develop sensitive tools for biomedical applications.

Keywords: Microarray technologies; Proteomics; Protein array; Protein interactions; High-throughput screening

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1. Introduction

Basic and applied post-genomic research requires high-throughput tools to elucidate the myriad of molecular interactions involved in complex cellular processes. Computational treatment of the massive amount of genomic information enables the properties of open reading frames (ORFs) and protein interactions to be predicted [1]. However, the necessity to validate the virtual data by experimental proof, to find answers for functions of unknown proteins and to establish protein interaction maps has stimulated the development of new technologies. It is clear that only complementary approaches will guarantee success when putting the acquired knowledge into practice. During the last decade, various technologies have been adopted for proteome-scale investigations. A great effort has been made in the large-scale 3D structural analysis of prokaryotic and eukaryotic proteins. This is seen as the best way to perform the structure-based assignment of protein functions and predict protein contacts with other molecules [2]. A high-throughput two-hybrid methodology, along with mass spectroscopy, has been largely exploited for the construction of the protein interaction maps of several organisms [3,4]. The emergence of array technology has been a logical consequence of this formidable scientific challenge to understand genome and proteome behavior in a whole unicellular or multicellular organism by the analysis of miniaturized spots composed of various biological materials.

Protein arrays have emerged as a transition from genomics to proteomics to study protein interactions with different types of molecular partner (Fig. 1). The advantages of this technology are based on its capacity to characterize a huge number of ordered protein spots simultaneously, thus replacing numerous individual binding reactions by a single test, and to monitor binding parameters in parallel assays with different probes [5]. Ordered protein spots can be arranged in planar macroarray or microarray formats, reflecting the relative size and number of spots per square centimeter to be studied; macroarrays are suited to the study of dozens of proteins whereas microarrays are suitable for the large-scale analysis of proteomes [6,7]. The success achieved in the analysis of the *Saccharomyces cerevisiae* proteome emphasizes the realistic possibilities of this technology to manipulate a bulk quantity of proteins [8].

The association of proteins with each other and with DNA leads to the formation of enzymatic and regulatory complexes that govern fundamental cellular processes such as transcription and translation, metabolic and signal transduction pathways, differentiation and development. Therefore, the elucidation of protein–DNA and protein–protein interactions is a clue both for understanding the nature of physiological and pathological processes in a whole organism and for creating new tools for basic and applied research. In this review, I present the achievements in the high-throughput production of functional recombinant proteins, the fabrication of protein arrays and the detection of protein–DNA and protein–protein interactions. I focus on the approaches that enable biologically active proteins to be obtained and the factors limiting the detection of specific molecular interactions to be overridden. These are the two crucial conditions for the development of protein array-based applications.

2. High-throughput protein production

Considering that the number of sequenced prokaryotic and eukaryotic genomes is continually increasing, one would expect that high-throughput protein production is indispensable for proteome-scale investigations of molecular interactions. Achievement of this goal depends on both the efficiency and the cost of the protein-producing methods. Neither antibody production nor peptide synthesis is discussed in this review (for recent reviews see [9,10]). Below, I present the approaches providing high-throughput protein synthesis in cellular and cell-free systems.

2.1. DNA cloning

High-throughput protein production in cells requires rapid DNA cloning and gene expression in prokaryotic and eukaryotic hosts. Gene cloning from sequenced prokaryotic genomes is rather simple to perform whereas cloning eukaryotic genes usually needs the creation of cDNA molecules. Comprehensive cDNA collections of eukaryotic genomes are currently available at several centers worldwide (http://www.ncbi.nlm.nih.gov/ncigap/, http://kazysa.or.jp/huge/). The cloned genes from cDNA collections can be expressed in certain host organisms or the coding sequences can be transferred into more suitable host-cloning systems by ligase-dependent and ligase-independent cloning (LIC) methods.

Traditional ligase-dependent DNA cloning involves the covalent connection of restriction endonuclease-generated

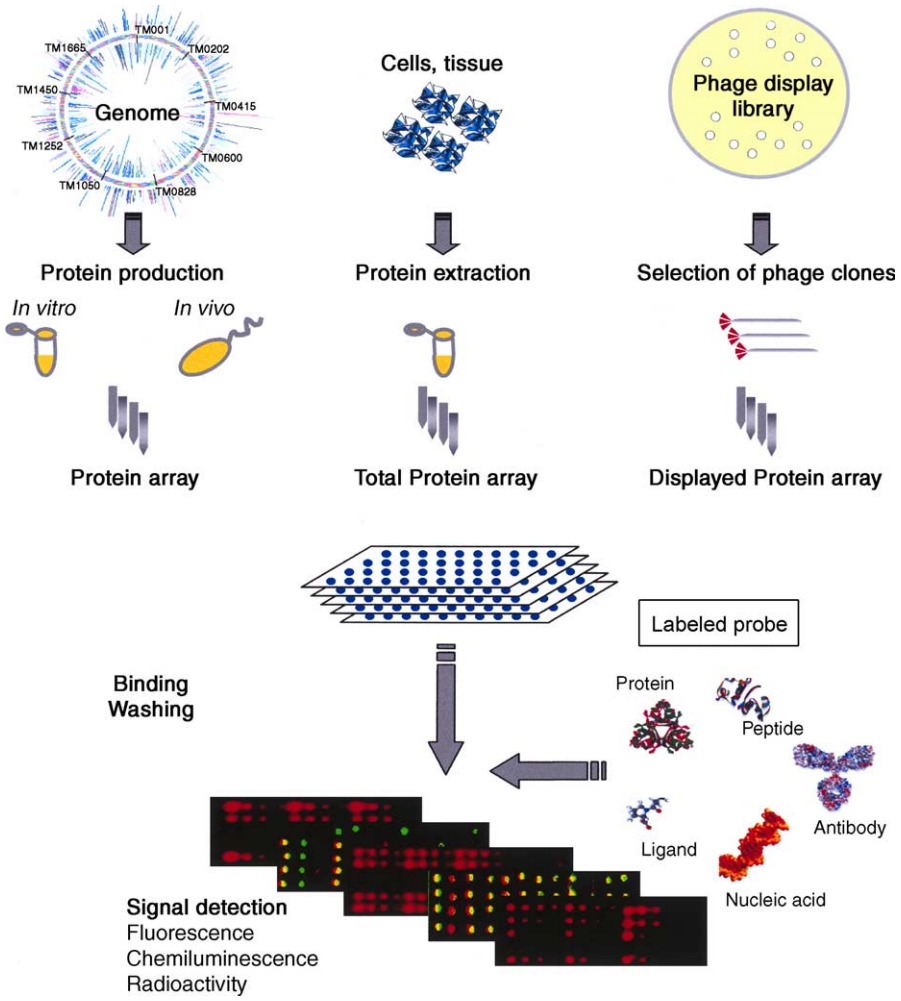

Fig. 1. Diversity of multiplexed and high-throughput protein arrays. The arrays can also be fabricated with monoclonal antibodies and chemically synthesized peptides. The figure shows an example of planar protein arrays and the fluorescent detection. The technology is applicable to study interactions with various types of molecules.

or PCR-amplified fragments to specialized expression vectors with polynucleotide ligase, followed by the selection of recombinant clones in appropriate hosts. The LIC methods eliminate the use of restriction endonuclease digestion and ligation of PCR products, thereby increasing the probability of successfully joining DNA molecules, simplifying gene insertion into a suitable vector and accelerating the DNA cloning steps in cells.

One of the first LIC methods demonstrated that 12-nucleotide overlaps in the target and vector PCR-amplified DNAs, generated by 3′ > 5′ exonuclease activity of T4 DNA polymerase, provide sufficiently strong annealing of complementary single strands [11]. Consequently, recombinant molecules can be selected in transformed *Escherichia coli* cells in which the cellular ligase catalyzes the phospho-diester links between the insert and vector DNAs. The method was later modified and several specialized vectors have since been constructed, which provide more efficient DNA cloning [12–14]. It is worth mentioning that, with the introduction of a two-step PCR-based overlap extension method without ligation, gene expression from a desired transcription–translation signal became a simple and a versatile procedure [15,16].

DNA topoisomerase from *Vaccinia* virus was found to be able to join DNA molecules in vitro in 5 min, a great advantage over the DNA-ligase catalyzed reaction [17]. Several well-known Topo vectors have been constructed for cloning blunt-end and cohesive-end generated DNA fragments using the topoisomerase activity (http://www.invitrogen.com).

Other LIC methods exploit in vitro or in vivo site-specific exchange between target and vector DNAs via recombination. The Cre-*lox* site-specific recombination system catalyzes in vitro fusion of the plasmid DNA carrying the target gene to another vector containing convenient expression and regulatory elements [18]. The popular Gateway system uses phage λ Int-Xis-IHF site-specific-mediated recombination, allowing the exchange of DNA regions flanked by recombination sites between two parental molecules [19].

However, the simplest means to generate exchange between DNAs is in vivo recombination between homologous sequences that can be detected by co-transformation of two linear DNA molecules and selection of recombinant clones in *E. coli* cells [20,21]. In this way, recombinant DNAs have also been selected in the *S. cerevisiae* host that possess a higher recombination frequency between homologous segments of linearized DNAs [22,23]. Recently, this method appears to have received a new impetus with regard to providing high-throughput DNA cloning to various sequenced genomes, as demonstrated by the successful cloning of almost 93% of ORFs from a pathogenic bacterium, *Campylobacter jejuni* [24]. Indeed, 20 bp tags created by PCR at the extremities of different linear DNA fragments are sufficient to assure efficient recombination with the homologous extremities of the vector DNA digested by two restriction endonucleases (digestion by two different enzymes eliminates the self-circularization of the vector DNA). After co-transformation of *E. coli*, almost 70% of antibiotic-resistant clones acquired the expected recombinant DNAs with cloned genes varying in size between 93 and 4020 bp. Western-blot analysis of total protein samples from recombinant clones confirmed that the majority of genes are expressed as fusion proteins, suggesting their usefulness for comprehensive functional studies [24].

2.2. Affinity purification and solubility of proteins

The purification of large sets of proteins should assure the maintenance of their three-dimensional conformation as this is a crucial condition to display both biological activity and recognition of molecular partners. The conversion of aggregated proteins into properly folded and biologically active molecules [25] appears not to be a convenient high-throughput approach because of the cost and uncertainty of the expected results. State-of-the-art protein production is based on affinity purification, which can be performed in a single step thereby accelerating the rate-limiting steps of protein purification by classical procedures. Moreover, affinity purification conditions can be standardized to provide the accurate purification of many representatives of different families of proteins.

Affinity binding to selected compounds can be achieved by relevant tags created at the N- or C-terminus of target proteins. The most popular affinity purification tags, His (usually six histidine residues) and glutathione-*S*-transferase (GST), bind to Ni-NTA agarose [26] and glutathione agarose [27], respectively. The GST-tag can improve the solubility of fused proteins, whereas some other tags can provoke the conversion of soluble proteins into insoluble molecules [28,29]. Certain proteins fused to the purification tags can be improperly folded, making the respective amino acid residues inaccessible for affinity binding [30,31].

Waldo and co-workers [32,33] suggested assessing soluble proteins with unknown functional assignments through the fusion of the corresponding ORFs to green fluorescent protein, GFP. Such a GFP-tag can be used as an indicator of protein folding, and fused proteins with high and low solubility can be identified by the fluorescence intensity of recombinant clones. This method has been used to characterize putative proteins encoded by random mouse cDNA fragments cloned in *E. coli* [34].

In spite of the absence of a universal affinity purification method and the difficulty of obtaining soluble proteins, remarkable results have been obtained in the simultaneous production of many proteins for structural and functional investigations. In fact, the requirement of milligram quantities of proteins for structural studies has stimulated the creation of high-throughput pipelines with the goal of resolving three-dimensional structures of representatives of all protein families with all possible folds. Non-membrane proteins of a thermophilic archaeon, *Methanobacterium thermoautotrophicum*, have been expressed in *E. coli* cells and 20% of 424 His-tagged purified samples were suitable candidates for NMR spectroscopic and X-ray crystallographic studies [35]. Almost 73% of ORFs have been cloned from a hyperthermophilic bacterium *Thermotoga maritima* (contains 1877 putative genes) and expressed in fusion to the affinity purification MGSDKHis6-tag at the N-terminus of proteins in *E. coli* cells [36]. The presence of six initial amino acids provided greater and more homogeneous expression of recombinant proteins without any significant influence on proper protein folding. Forty percent of the proteins tested were found to be soluble and 432 proteins were successfully crystallized, including some large proteins of 100 kDa (10^5 relative molecular mass).

Braun et al. [37] have performed a systematic study of the efficiency of high-throughput purification of human fusion proteins from *E. coli* under denaturing and non-denaturing conditions. Thirty-two full-length genes, coding proteins with a molecular weight range of 16–150 kDa, have been individually expressed in fusion to four affinity purification tags. Comparative analysis showed that a small His-tag and a 4 kDa calmodulin-binding peptide (CBP-tag) were useful to purify only a few proteins under non-denaturing conditions whereas larger tags, a 42 kDa maltose-binding protein (MBP) and a 26 kDa GST, provided purification of more proteins, with a purity in the range of 30–70%. Many impurities resulted from the degradation of recombinant proteins; GST-tagged proteins were found to be most prone to degradation. The purified GST- and MBP-tagged proteins were functional, as determined by kinase activation or kinase inhibition tests with corresponding candidates. Furthermore, 204 full-length proteins out of 336 random cDNAs have been expressed in fusion to a His-tag and purified under denaturing conditions. Remarkably, 192 GST-tagged proteins from this list of His fusion samples have also been successfully purified under non-denaturing conditions.

The expression of human proteins in fusion to seven different N-terminal tags in *E. coli* has been studied by Hammarström et al. [29]. Regarding the expression and the solubility of 26 tested small proteins (up to 19.5 kDa), a 13 kDa

thioredoxin, a 17 kDa double Z-domain from *Staphylococcus aureus* protein A, a 7.5 kDa Gb1 domain of *Streptococcus* protein G and MBP were found to be the best tags. Only 5.4% of the 86 selected His-fusion proteins, ranging from 10 to 110 kDa, were found soluble as compared to 24% of fusions to the MBP-tag [38]. Studying another 40 proteins from yeast, mammals, plants and insects confirms that proteins ranging from 9 to 100 kDa and purified in fusion to GST, and especially to MBP or NusA tags, display greater solubility [14].

These data emphasize that *E. coli* is still the best-exploited host system for the expression of recombinant proteins. However, its usefulness is limited to eukaryotic proteins, which do not require post-translational modifications for their biological activity. Besides, many multi-domain eukaryotic proteins appear not to fold correctly in the bacterial cytoplasmic environment because of differences in the coupling of translation and folding in eukaryotic and bacterial cells [39,40]. These barriers can be overcome by using eukaryotic cell hosts, as shown by the expression of 5800 ORFs of *S. cerevisiae* in a homologous host [41]. The proteins were fused to the tandem of GST- and His-tags, the first providing better solubility for target proteins and the second providing affinity binding to the Ni-coated glass surface for the preparation of protein arrays. In addition, both tags are suitable for affinity purification of proteins and detection by corresponding anti-tag antibodies. Other eukaryotic systems based on mammalian cells [42] and baculovirus ([43], http://www.protometric.com) are in development with the aim of using them for high-throughput production of human proteins.

A comparison of purified human proteins expressed as fusions to a tandem of N-terminal His- and GST-tags in *E. coli* showed that both tags provide a similar purity but the protein yield is higher in some cases on Ni-NTA agarose [44]. Double tags fused to both extremities of target proteins improve purification by the elimination of non-specific proteins and by decreasing the yield of truncated derivatives. Thus, more than 90% purity was reached for eukaryotic proteins fused simultaneously to the N-terminal GST- or MBP-tag and the C-terminal His-tag when two affinity columns were used [14]. Similarly, human proteins fused to the N-terminal His-tag and the C-terminal Strep-tag (the peptide possesses the affinity for streptavidin) and expressed in *S. cerevisiae* [45] or *Pichia pastoris* [46], or fused to the N-terminal GST-tag and the C-terminal Strep-tag and expressed in *E. coli* [47], have been purified with greater efficiency using the advantages of the two tags consecutively.

The data accumulated show that the small His-tag is suitable for the purification of recombinant bacterial and archaeal proteins, rather than for eukaryotic proteins under non-denaturing conditions. In this context, the GST and MBP tags are better suited to purify human proteins. However, the insertion of large tags increases the molecular masses of fused proteins and thereby decreases the efficiency of affinity purification [35,37,48]. Large tags, in particular MBP, can also affect the binding properties of target proteins so that a supplementary treatment of the proteins is needed to eliminate the tags and obtain biologically active molecules. Such a strategy appears to be unsuitable for massive protein purification. On the contrary, the development of small, efficient affinity peptide tags or alternative purification approaches might help to solve the problem and provide soluble eukaryotic proteins.

2.3. Cell-free protein synthesis

Proteins can also be synthesized in a cell-free system (a coupled in vitro transcription and translation system), first developed by De Vries and Zubay [49] with *E. coli* extracts known as S30 extracts. Intracellular metabolism, and especially membrane-related functions (respiration and transfer) of cells, are sensitive to the elevated concentrations of unusual heterologous proteins, which can result in the arrest of cellular growth. On the contrary, being devoid of the cytoplasmic membrane, the cell-free system is open and tolerant to overexpressed "toxic" proteins. Cell-free protein synthesis can be performed on both circular and linear DNA templates; it does not involve DNA cloning steps in cells and is therefore less time-consuming. Moreover, linear DNA templates are suitable to assess rapidly the effect of terminal tags on the protein function (Fernholz, personal communication). The short duration of synthesis and the reaction conditions are unfavorable for protein aggregation in cell-free systems. Consequently, a relatively high proportion of soluble recombinant proteins can be produced. The yield of cell-free synthesized proteins was significantly increased by using strong transcription and translation signals for gene expression and by supplying more suitable ATP-generating sources and compounds essential for biosynthesis [50–53].

We have used an *E. coli*-based cell-free batch system for the synthesis of 14 ORFs coding for putative regulatory proteins of the XylR (six proteins), LacI (five proteins) and GntR (three proteins) families using PCR-amplified DNA templates from a hyperthermophilic bacterium *T. maritima* [54]. The heat treatment of the reaction products and further purification of N-terminal His-tagged proteins in microplate wells coated with Ni-NTA or containing corresponding magnetic beads allowed high-purity soluble proteins to be obtained with molecular masses from 14 to 43 kDa. The quantity of proteins synthesized under batch conditions was sufficient to test the binding to DNA by gel-mobility shift and macroarray methods.

Twenty-four proteins from several mesophilic microorganisms have also been fused to the His-tag and expressed in an *E. coli* cell-free system using circular plasmid DNA templates [55]. No difference was observed in the synthesis of 21 out of 24 proteins of 11.7–44.6 kDa at 25 and 30 °C. Colorimetric detection of soluble non-purified proteins by dot-blot using anti-His monoclonal antibody indicated that the C-terminal His-tag appears to decrease the protein solubility as compared to the N-terminal tag. However, more extensive experiments showed that the C-terminal His-tag is better for protein solubility (Fernholz, personal communication).

Eukaryotic cell-free protein synthesis systems have also been developed using extracts of reticulocytes and wheat germs [56,57]. The elimination of endogenous translation inhibitors and traces of ribonucleases, deoxyribonucleases and proteases in wheat embryos [58] and the optimization of the 5′- and 3′-untranslated regions, along with the stabilization of mRNA templates [59,60], allowed an improved in vitro process to be proposed, providing the synthesis of eukaryotic proteins from PCR-amplified DNAs. In fact, between 0.1 and 2.3 mg/ml of 50 *Arabidopsis thaliana* proteins ranging from 11.3 to 82.5 kDa were synthesized in 36 h by this cell-free system [59]. The presence of an N-terminal GST-tag increased the solubility of certain proteins considered as insoluble without this tag. Four of the five tested kinase proteins possessed autophosphorylation activity, suggesting that their kinase domains were folded into active forms.

In parallel with batch synthesis, more productive continuous flow cell-free translation systems have been developed in which the compounds necessary for synthesis are supplied to the reaction chamber through a semi-permeable membrane [61–64].

2.4. Automation of protein production

LIC methods, cellular and cell-free protein synthesis and affinity tag purification of proteins are amenable to automation, which should improve the efficiency and reduce the total cost of the high-throughput analysis of proteins. Indeed, 96-well formats have been successfully employed to clone many target genes simultaneously, to produce recombinant plasmids, to purify proteins by affinity chromatography and to study protein solubility and enzymatic activity [2,34,36,44,47,55,65,66].

An active human single-chain anti-progesterone V_H/K fragment fused via a flexible linker to the C-terminal His-tag has been synthesized in 96-microplate wells, using rabbit reticulocyte lysates, and immobilized from extracts to a Ni-NTA coated surface in the same wells [67]. Furthermore, a highly productive continuous flow cell-free system, known as rapid translation system (RTS), supported by ProteoExpert software to optimize protein expression has been commercialized. It provides the simultaneous synthesis of prokaryotic and eukaryotic proteins in 48 small independent semi-permeable bioreactors of 50 μl volume (http://www.biochen.roche.com).

Taking into consideration the advantages of cell-free protein synthesis, it is expected to become a powerful means for high-throughput production of biologically active proteins that can be perfectly integrated into the fabrication of protein arrays.

3. Supports for arraying proteins

Miniaturized protein arrays are fabricated on supports, which must satisfy strict requirements. These include providing good quality spots, a low background, simplicity of manipulation and compatibility with detection systems. In binding reactions on microspot-formats, the capture proteins are covalently or non-covalently immobilized on a planar surface whereas the molecular partner(s) is in a solution (see Fig. 1). Alternative supports and formats have been developed, which ensure the arrayed proteins are closer to their native state.

3.1. Oriented immobilization

The glass slide is an inert and mechanically stable support, which requires a chemical coating to make it functional. The chemistry of immobilizing target proteins on a glass slide is based on either the non-covalent binding of proteins or covalent bonding between amino acids or carbohydrates attached to the proteins and the functional groups of chemical agents previously fixed on the slide. A comprehensive list of glass slide surfaces chemically modified for the immobilization of proteins has been recently presented elsewhere [68].

The direct and random immobilization of proteins on a solid phase limits their number, causes their denaturation and, consequently, reduces the specificity of the molecular interactions [69]. Polyethylene glycol (PEG) can form a monolayer that reduces non-specific attachment of proteins on a solid phase [70]. Therefore, poly-L-lysine- or epoxy-grafted PEG slides prevent direct contact between the proteins in solution and the glass surface thus decreasing the background [71,72].

A more effective strategy is the oriented immobilization of target proteins, which enables a considerably greater quantity, and thus more functional capture proteins, to be deposited. As a result, a proportionally larger number of molecular partners in the liquid samples analyzed can be captured as first demonstrated by immobilization of antibodies through their carbohydrate groups on a solid phase [73,74]. Cross-linkers can also be used to improve both the immobilization of antibodies on the surface of the glass slide and their orientation towards the antigens in solution. The insertion, between the antibodies and the support, of a $(CH_2)_n$ radical (single or extended by other radicals) with ends that bind to the amine or thiol groups of the antibodies, can facilitate the access of antigens to captures [75]. This approach gives the best performance on glass slides derivatized with 2.5% epoxysilane. When the immobilization of differentially biotinylated immunoglobulins IgG or Fab fragments on a layer of streptavidin were compared, the ability of oriented antibodies to capture analytes was found to be 5–10 times higher than that of non-oriented molecules [76]. Biotinylated antibodies and peptides were also immobilized on an oriented streptavidin monolayer self-assembled onto a gold film [77].

Biotinylation of other proteins is less specific when compared to antibodies. However, this drawback was overcome by using an in vivo intein-mediated expression system to generate a C-terminal intein-tag with a chitin-binding domain [78,79]. The fused protein was purified on a chitin column by affinity. Then the column was flushed with biotinylated cys-

teine and the C-terminally biotinylated protein, after spontaneous liberation from the intein-tag, could be site-specifically attached to avidin-functionalized glass slides. A more general principle is the affinity immobilization of proteins containing the His-tag on a glass surface coated by nickel [8] or the GST-tag on glutathione-derivatized glass slides [80]. This simple approach appears to be particularly suitable for the immobilization of proteins from organisms whose genomes have been sequenced because affinity tags can easily be introduced by PCR upstream or downstream of coding regions.

3.2. Hydrogel

Another technique that can increase the efficiency of protein immobilization consists of covering the glass slide with a layer of a polymer, such as polyacrylamide, agarose or gelatin, which provides a porous structure. A film of gel, which is 70–95% water, ensures that the three-dimensional structure of the immobilized proteins is maintained and their accessibility is favored without them having to be oriented [81]. Moreover, the low threshold of fluorescence of this type of support leads to a greater sensitivity of detection. The proteins can be placed in solution with polyacrylamide and co-polymerized by UV irradiation [82] or spotted directly onto a layer of commercial hydrogel (http://www.perkin.elmer.com). The immobilized enzymes retain almost 70% of their activity.

The supramolecular hydrogels that possess amphiphilic characteristics have been recently developed for semi-wet protein microarrays [83]. They contain aqueous pockets created in the gel matrix where enzymatic reactions can occur and hydrophobic spaces to trap the fluorescent products released during the reactions. These supports thus appear to be promising for the direct monitoring of enzymes and the screening of their substrates and inhibitors.

3.3. Nitrocellulose membrane

The nitrocellulose membrane is suitable for the immobilization of different proteins and is easy to manipulate. It has therefore been successfully applied to the fabrication of protein arrays and to studies of various molecular interactions [6,84]. Although hydrophobic interactions are generally considered as responsible for the immobilization of proteins, the actual forces involved in their binding are not yet known. Due to its microporous surface, nitrocellulose is able to retain a greater number of capture proteins than a planar surface of aldehyde-derivatized glass slides [85]. Moreover, its porosity contains, under appropriate conditions, aqueous microspaces that allow proteins to maintain their active configuration. As the half-life of the immobilized proteins is longer, this type of array can be used for several months. In addition, the new nitrocellulose membranes, of the FAST-slides type, have a higher signal-to-noise ratio which markedly improves detection by fluorescence ([86], http://www.schleicher.schuell.com).

Proteins are usually purified before being deposited. However, porous supports, like nitrocellulose membranes or hydrogel, are equally compatible with crude cell extracts containing non-purified overexpressed proteins [82,87].

3.4. Other supports and micro-formats

A chemically modified surface of conventional microplate wells has been used to fabricate protein microarrays. Several geometric configurations of up to 48 spots per well have been proposed. Such a double microplate–microspot approach appears to have an advantage in terms of performing high-throughput analysis in individual chambers [88,89]. Alternatively, microfluidic chips have been developed for multiple enzymatic and binding reactions [90,91]. The surface plasmon resonance (SPR) sensing technique enables the equilibrium binding constants of molecular interactions to be measured in real time without the need for labeled probes [92]. This measurement is now possible for 400 individual spots simultaneously, which increases the value of SPR for the high-throughput screening of molecules (http://www.applied.biosystemes.com).

In order to avoid dehydration and denaturation of proteins, binding reactions can be carried out between non-immobilized free capture proteins and analytes in small individual chambers. Such microwells have been successfully used to detect the kinase activity of many putative yeast proteins [41]. A better performance of enzymatic reactions can be achieved in small nanowells developed in recent years [93,94]. Moreover, an active β-galactosidase has been synthesized in a bacterial cell-free system in a volume of 0.1 μl, sufficient for many enzymatic measurements [95]. Therefore, nanowell-based arrays will be useful to identify catalytic functions promoted by non-immobilized proteins synthesized in vitro in the same wells.

The majority of membrane proteins, which possess particular folds, require a bi-lipid environment to display a biological function. Consequently, the array method has been successfully employed to detect interactions of recombinant GABA receptors with a small carbohydrate by immobilization of a neuronal membrane fraction on nitrocellulose [96]. Remarkably, artificial bi-lipid layers have been developed on microplates coated by an amine-presenting surface that allows both the three-dimensional structure and the binding capacity of the arrayed G-protein-coupled receptor proteins to be conserved [97].

This diversity of supports and formats widens the potential use of array-based methodology to study interactions of different families of proteins. The high quality of planar supports, which are the most suitable for simultaneously screening molecules, contributes significantly to the binding specificity to both small ligands and large macromolecules under appropriate conditions. Further development of supports with improved characteristics will provide biological activity of unstable proteins and those requiring particular compartments on a minute area.

4. Detection of signal

Several detection methods have been developed to monitor signals with protein arrays using labeled and non-labeled molecular probes. Three of them, being the most sensitive, are widely exploited to study interactions of proteins arrayed on planar surfaces. Radioactive detection requires fewer binding/washing steps but the spot resolution is relatively low (>300 μm) and the technique is demanding in terms of staff protection. Chemiluminescence detection is limited in performance because of signal instability. Stable fluorescent signals of different wavelengths, which depend on the absorption and emission of photons by a given fluorophore and its ability to undergo numerous excitation–emission cycles, are better suited for detection on the minute area of spots. Moreover, they can be used to monitor different functions simultaneously in two-color binding experiments.

4.1. Protein labeling by cell-free synthesis

In fluorescence detection, protein labeling is carried out by bioconjugation, i.e. by covalent coupling of mono- or bifunctional groups of fluorescent dyes to primary amines of the N-terminus or other active groups in some amino acids (usually an ε-NH_2 group of lysine). Amine-reactive dyes are used to label proteins, oligonucleotides and other biomolecules, whereas thiol-reactive dyes are used to label selectively cysteine residues in proteins. A drawback of these molecular probes is that the dyes conjugated randomly to amino acids can induce topological changes in the protein structure that might affect the recognition of molecular partners. A common way to avoid this inconvenience is to apply the "sandwich principle" for the detection of bound molecules via a supplementary binding step of the analyte to a primary antibody that is recognized by a labeled secondary antibody. However, cell-free protein synthesis opens up a new possibility of introducing a single fluorescent molecule precisely into the N- or C-terminal position of a given protein.

One of these methods uses low concentrations of puromycin at which the antibiotic competes with aminoacyl-tRNA for incorporation into the C-terminal of de novo synthesized protein [98,99]. Up to 90% of the synthesized protein can be labeled with puromycin bearing a fluorophore [100] without significant perturbation of DNA binding and other biological functions [101].

Another method is based on the observation that an initiator tRNA with an altered anti-codon can substitute methionine by another amino acid at the beginning of a protein [102]. Indeed, the N-terminus of model proteins has been labeled with an efficiency of up to 67% by using an amber (CUA) initiator tRNA, chemically aminoacylated with a fluorophore-amino acid conjugate, and the DNA template containing the amber UAG codon instead of the AUG initiation codon in the *E. coli* cell-free system [103,104].

Thus, there is a great opportunity for array technology to acquire uniformly labeled and correctly folded proteins for probing protein–protein interactions.

4.2. Enhancing signals from microspots

Protein microspots on a planar support provide higher sensitivity for the detection of interactions with molecular partners as compared to other binding-format assays. The theoretical basis of miniaturized ligand binding assays has been developed by Ekins and coworkers [105–107]. Assuming that no dissociation and rebinding takes place under standardized conditions, the detected total signal on microspots reflects the number of molecules bound by capture proteins. However, if the surface density is identical in a minute area, then the spot of a smaller diameter provides a stronger signal than that of a larger diameter.

In practice, the signal detection from spots is limited by the number of capture proteins: below a critical threshold, current methods fail to detect bound molecules. Since the sensitivity of the detection depends on the surface density of functional capture molecules, the orientated immobilization of proteins on derivized glass surfaces increases the total protein surface exposed to the interactions and thereby enables the detection of a lower number of capture proteins [8,75,76]. A greater surface contact of capture proteins can also be achieved by their immobilization on 3D space-supports, such as hydrogel or nitrocellulose (see Section 3).

Alternatively, the critical threshold of the signal to be detected can be lowered by enhancing the signal-to-noise ratio on a given support. Indeed, the sensitivity of fluorescence detection is diminished by background signals, which can be caused by endogenous constituents of the supports or the samples (usually referred to as autofluorescence). Visible fluorophores, which absorb and emit within ≈500–650 nm, are not well suited to detect proteins on a nitrocellulose membrane because of the high background fluorescence of the support in the visible range. In contrast, near-infrared fluorescent dyes (IRDyes), with wavelengths higher than 670 nm, have a great advantage over visible fluorophores in that longer wavelengths provide very low background (high signal-to-noise ratio) and a wide dynamic range to detect proteins on membrane supports [108]. IRDye-labeled antibodies allow membrane-immobilized proteins to be detected with higher sensitivity than those labeled by visible fluorophores ([109], http://www.licor.com). Moreover, attomolar quantities of target proteins have been detected in serially diluted samples arrayed on a nitrocellulose membrane using IRDye-labeled DNA and protein probes [87].

Another radical way to increase the sensitivity of detection on arrayed microspots is by signal amplification. The tyramide amplification method, based on a sandwich principle, uses the horseradish peroxidase-mediated activation of multiple copies of fluorescent- or chemiluminescent-labeled tyramide derivatives to generate high-density labeled proteins (http://www.probes.com). The method was successfully ap-

plied to screening pathway targets in tumor tissues by reverse-phase protein arrays [110,111]. This amplification approach also enabled the detection of low-abundance IgEs against some allergens used as immobilized antigen arrays [112].

PCR technology has also been used to detect antibodies bound to antigens via the attachment of a specific DNA sequence as a target for signal amplification [113–116]. A DNA molecule can be connected to a given antibody by streptavidin–biotin or covalent linkage. Furthermore, a method has been developed known as rolling circle amplification (RCA) [117] that can detect low-abundance antigens on microspot formats of arrayed antibodies [118,119]. In this method, a reporter antibody conjugated to the 5′ end of an oligonucleotide is first captured by an antigen immobilized on a support. Then this oligonucleotide in the bound antibody–antigen complex is annealed to a complementary sequence located in a small circular single strand of DNA (≈80-mer) and the added DNA polymerase produces a long linear concatamer molecule by repeated rolling of the circular DNA template. Finally, the resulting amplified DNA, containing hundreds of copies of the circular DNA and attached to the bound antibody-antigen complex, is detected by hybridization with a labeled complementary oligonucleotide probe or by direct incorporation of fluorescently-labeled or hapten-labeled nucleotides. This elegant method provides linear kinetics of DNA amplification by incorporation of ≈200 nucleotides/s with a 3 log increase in the detection sensitivity, up to zeptomolar quantities of some target proteins. Thus, 0.1 pg/ml of the prostate-specific antigen was detected by this method as compared to 100 pg/ml by conventional ELISA. RCA has been used for multiplexed detection of low-abundance proteins in biological fluids on microarray formats, such as cytokines secreted during maturation of Langerhans cells [120] and allergen-specific IgEs in the sera of patients [121,122].

Another possibility for signal enhancement is based on the proximity-dependent DNA ligation of two affinity DNA aptamer probes which, being bound to target proteins and hybridized to a connector oligonucleotide, serve as templates for signal amplification by PCR [123,124]. Without additional washing and separation steps, up to 40 zeptomole amounts of the cytokine platelet-derived growth factor have been detected by this extremely sensitive method. It may prove suitable for enhancing the sensitivity and specificity of the detection of multiple protein partners using array technology.

Nanomechanical detection of a bent cantilever with the immobilized protein bound to the analyte is also promising for the development of label-free monitoring of molecular interactions on protein arrays by the application of atomic force microscopy [125–127].

5. Molecular interactions

The combination of computational and molecular biology approaches has already proven its power to detect functional motifs in protein and DNA sequences and to identify complex regulatory networks in cells. A high-throughput yeast two-hybrid method based on a “bait-prey”-promoted activation of a reporter gene in cells [128] has been largely used for the construction of interaction maps of several organisms [23,129–133]. Furthermore, mapping of protein complexes by mass spectroscopy has been reported [134,135]. The emergence of a multiplexed high-throughput array technology raises studying protein–DNA and protein–protein interactions to a new qualitative level by providing both complementary information and the unique possibility of analyzing genomes and proteomes of unicellular and multicellular organisms.

5.1. Protein–DNA interactions

Many DNA-binding proteins are involved in the regulation of genome expression and maintenance. The majority of DNA-binding regulatory proteins are two-domain molecules, in which one domain usually determines DNA recognition and binding, while the other is involved in the assembly of monomers into oligomeric molecules and the recruitment of cofactors and other proteins to regulate RNA synthesis [136]. The target sites for each family of proteins represent similar nucleotide sequences that share a core motif and transcriptional regulation is specified by various factors that affect the efficiency of base pair-amino acid contacts in cells.

Sensitive assays have been developed to identify protein–DNA interactions using fluorescence spectroscopy, nuclear magnetic resonance, mass spectroscopy, and atomic force microscopy during recent years [137–144]. Protein binding sites can be predicted in genomes by bioinformatics, although this approach alone does not provide valuable functional information [145,146]. A convenient way to assess simultaneously a dozen proteins binding to DNA targets is still the gel electrophoresis mobility shift assay (EMSA). In this context, the array technology has significant time and cost advantages over other DNA-binding methods, which can take months to characterize and to assess a large number of natural and mutant targets of interest.

Several DNA array-based approaches have been developed to monitor DNA-protein interactions. The first high-throughput analysis was performed by enzymatic conversion of single-stranded oligonucleotides, which contain a longer 16-mer sequence for annealing, into double-stranded oligonucleotides that were then probed to bind transcriptional factors [147]. Different parameters of the method have been improved by immobilizing shorter 8-mer oligonucleotides on polyacrylamide gel pads, and consecutive annealing with a specified mixture of 8-mer oligonucleotides [148] or by forming unimolecular hairpin structures [149]. The approach has also been used to analyze the specificity of zinc finger interactions to wild-type and mutant nucleotide sequences using phage-displayed peptides [150,151] and to characterize the single nucleotide polymorphism effect in human transcriptional factors NF-κB and OCT-1 [152].

A combined method of DNA microarray and chromatin immunoprecipitation (ChIP array) has been developed to assess the binding of gene-specific transcription activators in response to changes in carbon source and mating pheromone in the yeast genome [153,154] and to detect binding loci for cell-cycle regulators, the p65 subunit of NF-κB transcriptional factor and the cyclic AMP-responsive element-binding protein on human chromosomes [155–157]. A similar approach uses the separation of bound protein/DNA complexes by EMSA instead of immunoprecipitation [158]. Another method, DNA adenine methylase identification (DamID array), detects DNA-binding sites by taking into consideration that eukaryotic DNA can be subjected to methylation in vivo only at chromosomal sites bound to a given transcription factor fused to the bacterial Dam enzyme [159,160].

The DNA microarray method has recently been combined with SPR microscopy to monitor the binding constants of the yeast transcription factor Gal4 with respect to many target DNAs prepared by spotting biotinylated dsDNA on a streptavidin-coated gold surface [161]. The kinetics of the binding of the proteins to operator sequences was monitored simultaneously from 120 spots with a sensitivity of 0.5 pg of bound protein at a time resolution of 1 s.

While DNA arrays allow DNA binding sites in genomes to be identified and their response to growth conditions to be assessed, protein arrays can help to identify proteins that bind to DNA targets of interest.

In the "proof-of-concept" study, Ge developed a universal protein array (UPA) system for the quantitative detection of protein interactions with different molecules [6]. The proteins were overexpressed in bacteria or in baculovirus and purified to homogeneity. A macroarray representing 48 individual patterns was prepared on a nitrocellulose membrane with dot blot apparatus. The majority of the chosen proteins were general or specific transcriptional factors potentially enabling binding to DNA, RNA, other proteins and/or small ligands. Proteins were probed to a ^{32}P-labeled 64 bp double-stranded or single-stranded oligonucleotide containing the major late promoter region of the adenovirus. A tight binding was detected between the phosphorylated transcriptional activator PC4 and dsDNA, whereas the substitution of a single amino acid that is crucial for the DNA-binding activity of PC4 [162] completely abolished the binding ability. The feasibility of UPA was confirmed with other proteins as well [6]. This pioneering work emphasizes both the high-throughput and multiplexed character of protein array technology for the simultaneous analysis of many protein interactions using various molecular probes.

We used protein microarray in combination with IRDye fluorescence detection to assess protein–DNA and protein–protein interactions on a nitrocellulose membrane [87]. Arrays prepared from serial dilutions of several purified proteins and crude extracts containing the proteins overexpressed from a T7 promoter after IPTG induction were probed to the DNA promoter-operator region P*argCo* from

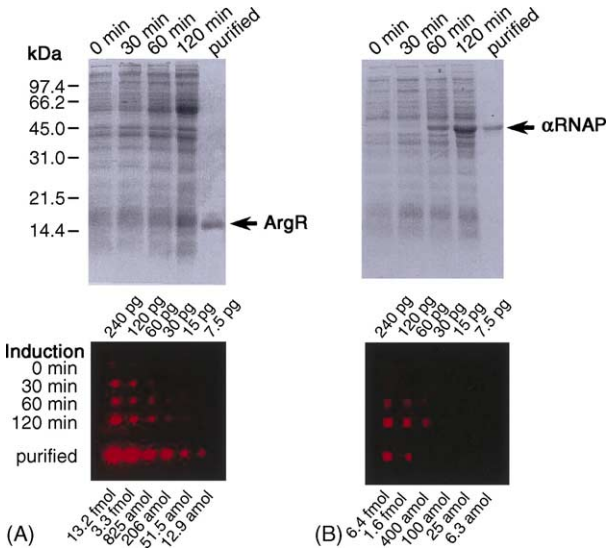

Fig. 2. SDS–PAGE analysis and fluorescence detection of protein–DNA interactions with arrayed *B. stearothermophilus* ArgR repressor (A) and *E. coli* RNA polymerase α subunit (B). Total proteins (crude extracts) of non-induced and 30, 60 and 120 min IPTG-induced samples were loaded on a polyacrylamide gel. The last lane contains a purified His-tagged protein. Arrays were prepared with the same crude extracts by a serial two-fold dilution and with pure proteins by a serial four-fold dilution. Total protein in spotted cell extracts is shown in pg while the amount of spotted pure proteins is shown in fmol and amol. Binding reactions were carried out with a 76 bp IRDye-800 labeled DNA carrying the *B. stearothermophilus* P*argCo* promoter-operator region (reproduced from [87] with permission).

Bacillus stearothermophilus carrying the expected binding sites. Binding signals were detected for up to 1.6 fmol and 12.9 amol of purified α subunit of RNA polymerase and ArgR repressor, respectively (Fig. 2). Moreover, a linear increase in a fluorescent signal was observed in crude extracts as a function of the duration of IPTG-induction, showing that the method works even with non-purified proteins. No signal was detected with shorter DNA probes that had lost the corresponding binding sites. Protein–DNA and protein–protein interactions with Kd of the order of 10^{-8} to 10^{-7} M could be detected with immobilized purified proteins and with crude extracts. This emphasizes the extreme sensitivity and the superiority of the IRDye detection of molecular interactions on membrane supports.

Next, this highly sensitive method was applied to a comprehensive analysis of the ArgR-mediated regulatory system in distant mesophilic and thermophilic bacteria [163,164]. In ArgR proteins, a winged helix-turn-helix motif (wHTH) located in the N-terminal DNA-binding domain [165,166] recognizes two adjacent 18 bp imperfect inverted repeats (Arg boxes) in operators [167,168], whereas distinct amino acids located in the C-terminal domain are responsible for the binding of L-arginine co-repressor and protein oligomerization [169]. Domain- and linker-replaced chimeric proteins were constructed from *E. coli* and *Bacillus stearothermophilus* repressors and eight proteins were compared in parallel assays

(64 spots of two-fold diluted samples on each membrane) to bind operator DNAs in the presence and the absence of arginine [163]. The detected signal intensity correlated with the DNA-binding affinity as confirmed by EMSA and SPR. Moreover, the ability to visualize and assess simultaneously fluorescent signals from all spots allowed us to reveal that the affinity for the operator site depends on the source of the DNA-binding and oligomerization domains in chimeras. Further detailed analysis of arrayed wild-type and mutant proteins from *E. coli*, *B. stearothermophilus* and *T. neapolitana* showed that bacterial ArgRs can differentially bind to operator targets composed of a single and double Arg-boxes [164]. The data obtained with protein arrays and other methods indicated that arginine regulatory proteins of the wHTH family can be of different kinds. At one extreme, there is a global kind of regulator from *T. neapolitana* possessing low repression efficiency and poor target specificity (binding to a single Arg-box in the absence of arginine). At the other extreme, there is an *E. coli* repressor exhibiting strong arginine-dependence and high target specificity (binding to a double Arg-box in the presence of arginine). Other ArgR regulators, from *Bacillus* for example, appear to be somewhere in between.

The question arises as to whether protein array technology is suitable to compare simultaneously regulatory proteins deduced from sequenced genomes and to identify potential DNA-binding sites in multiplexed assays. As a first step in that direction, we prepared a macroarray with immobilized cell-free synthesized *T. maritima* proteins belonging to the XylR, LacI and GntR families ([54], see Section 2.3). Thermostable proteins were probed to the characterized operator sequences from the *E. coli* genome. Fluorescent signals were detected for some of XylR, LacI and GntR proteins as an indication of their functionality. Moreover, the binding ability of these putative regulators to heterologous operators was confirmed by EMSA.

This method has recently been applied to assess the human serum response factor binding to wild-type and mutant DNA sites [170]. A 16-fold higher affinity was detected for a wild-type binding site as compared to the mutant site with a sensitivity of 0.4 pg of the spotted protein. As a further effort in studying human protein–DNA interactions, we have immobilized commercially available human transcriptional factors on a nitrocellulose membrane and probed to short DNA fragments containing putative binding sites predicted by bioinformatics within large promoter regions of human *Nat1* and *Nat2* genes (Yeretssian, Sakanyan, unpublished data).

The increasing interest in recombinant transcription factors as potential therapeutic agents and in artificial peptide–nucleic acids as potential modulators of protein–DNA interactions [171–174] faces several important issues including the high-throughput screening of biomolecules. Although limited, the data obtained with protein arrays are encouraging and show the effectiveness of protein array technology in the analysis of protein–DNA interactions and in the search for transcription activators/inhibitors.

5.2. Protein–protein interactions

Understanding the complexity of the protein–protein interactions involved in various cellular processes is a major goal of modern biology. The number of possible contacts between protein surfaces is astronomical although, in biological systems adapted to particular physiological conditions during evolution, this might be limited to interactions that provide a coordinated structure–function relationship between proteins necessary for surviving organisms. Only the combined efforts of bioinformatics and high-throughput techniques will help to find solutions in this attractive field of proteomics, or at least to contribute to the resolution of the most significant biomedical problems. In this context, protein array technology opens up wide perspectives to characterize putative proteins and identify molecular partners involved in metabolic and regulatory networks in cells. The advantages of detecting directly protein–protein interactions and monitoring different parameters such as relative protein concentration, binding affinity and protein modifications impart to protein arrays a particular usefulness for new applications.

5.2.1. Functional analysis by protein arrays

The first studies to use the high-throughput array approach were directed at identifying proteins by screening clones in a random library of human fetal brain cDNA using well-characterized antibodies [175,176]. However, protein interactions determine the transitory stability of associated functional complexes, which suggests the possibility of identifying and characterizing a suspected protein through its interacting partner with a known function.

Protein phosphorylation is a general and important mechanism of cellular regulation that involves at least two protein partners [177] therefore it was attractive to target this mechanism by protein arrays [7]. The substrate specificity of kinases can be studied on arrays with radioactive ATP or fluorescent-labeled antibodies specific to phosphorylated amino acids [7,178,179]. Snyder and co-workers [41] demonstrated the reliability of protein microarrays in determining the functions of putative kinases deduced from a sequenced genome. One hundred and nineteen GST-fused proteins of *S. cerevisiae*, predicted as candidates for kinase activity, were immobilized on a silicone surface in microwells and tested with 17 different protein substrates using ^{32}Pγ–ATP under appropriate conditions. Phosphorylation signals were monitored from radioactive spots by a high-resolution phosphoimager. The substrate specificity of a given enzyme was detected by normalization of a particular activity against all substrates. This study allowed the affiliation of the identified new enzymes to kinase families to be determined and a phylogenetic tree of proteins carrying the kinase core domain to be proposed.

The high-throughput and multiplexed functional analysis of putative proteins was extended to study 5800 *S. cerevisiae* putative proteins (93.5% of total ORFs) fused to the GST-His tag at their N-termini and purified from yeast cells [8].

Protein microarrays were fabricated by immobilization of protein patterns on aldehyde-treated or nickel-coated slides and tested for protein–protein and protein–lipid interactions. Calmodulin, a calcium-binding protein involved in various calcium-regulated processes, was found to bind 39 proteins, 33 of which were considered as new potential partners. Sequence alignment of these proteins identified a common motif in 14 calmodulin-binding proteins. Obviously, this comprehensive study exceeds the bounds of a global analysis of the yeast proteome and points towards similar investigations of entire proteomes from other organisms in the future.

The highly ordered chromatin–nucleosome structure forms numerous protein complexes, which directly or indirectly determine the functional state of DNA replication, reparation and transcription. Therefore, a protein microarray was designed to analyze the chromatin-related protein interactions of immobilized histones and other relevant proteins [180]. Probing to several DNA reparation proteins responsible for distinct functions showed that Rad51B interacts with histones and not nucleosomes. On the contrary, Smarcal1, a new member of the SWI/SNF family of proteins involved in nucleosome remodeling, bound specifically to nucleosomes and not histone proteins.

Remarkably, the microarray experiment for studying protein–protein interactions can be designed completely from cell-free synthesized proteins. Thus, five synthesized eukaryotic proteins spotted and mutually probed, after being labeled in vitro by the puromycin method, formed the expected interacting pairs [80]. Several cell-free synthesized *T. maritima* transcription factors arrayed on a nitrocellulose membrane were found to bind to the bacterial RNA polymerase alpha subunit [54]. Protein binding information can also be obtained from spotted non-purified cell extracts, as performed when studying interactions between the alpha subunit of *E. coli* RNA polymerase and the cyclic AMP receptor protein [87].

The functional studies of protein–protein interactions can be enlarged and diversified by the combination of protein array and phage display methods (see Fig. 1). Particularly noteworthy is the use of the array approach to screen recombinant antibodies in phage displayed clones, robotically picked and gridded on a support, to identify antibody fragments against purified antigens or impure proteins [181].

We have recently fabricated a protein microarray format of phage-displayed peptides to evaluate the immune response in HIV-1 infected patients and to compare protein–antibody interactions of epitope-mimicking peptides [182]. 18 different peptide sequences, reminiscent of the gp41 immunodominant epitope, CSGKLIC, that forms a typical disulfide-bonded loop [183] and was used for the immunological diagnosis of HIV-1 [184], were compared in the binding affinity for IgG of several patients in parallel assays. Substitutions of variable amino acids within the motif and adjacent sequences affected the ability of HIV-specific antibodies to recognize the epitope (Fig. 3). A good correlation was observed between the binding strength distribution data obtained by ELISA and by mimetic peptide microarray. However, the more sensitive, rapid and less compound-consuming phage peptide microarray format appears to be better suited to detect sub-optimal antigen–antibody interactions in a high-throughput and multiplexed manner. This study indicated that the antiviral therapy could lead to a decrease in the level of gp41 immunodominant epitope-specific antibodies as a result of the accumulation of HIV-1 mutants with decreased affinity for primarily generated antibodies [182].

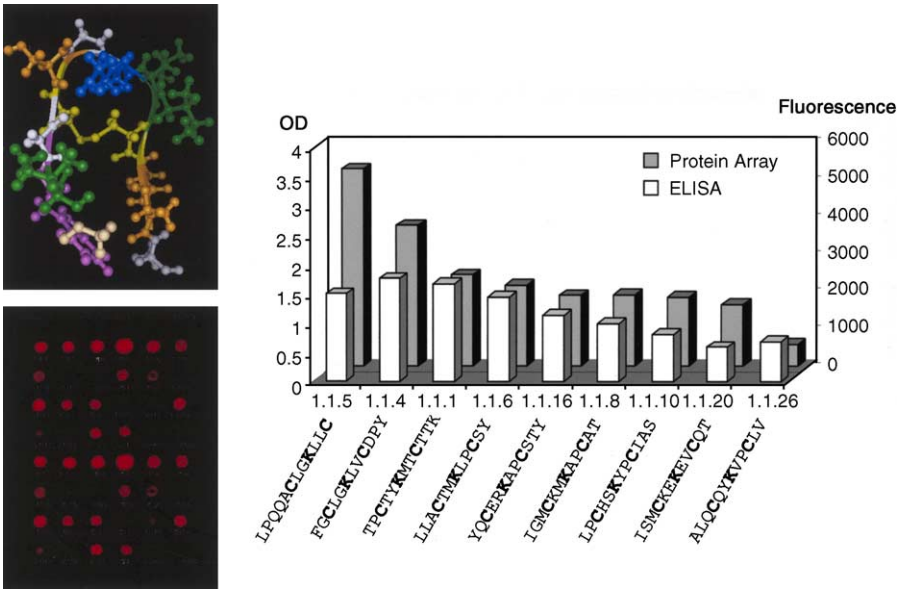

Fig. 3. Antibody-binding reactivity of phage-displayed mimetic peptides monitored by antigen array and microplate ELISA methods using IgG purified from an HIV-1 infected patient (adopted from [182] with permission). The 3D structure of the immunodominant epitope (IWGCSGKLICTTA) is from [183]. The conserved amino acid residues in selected peptides are shown in bold. An example of the ordered array with immobilized phage particles is shown.

Microarray-based detection of antigen–antibody interactions has been used for the functional assessment of proteins and peptides as potential antigens, the evaluation of the immune response and the profiling of antibodies in sera [88,121,185–192]. Therefore, antigen microarrays have a particular interest for medicine in the prognosis and diagnosis of different pathologies such as allergies and the autoimmune response, cancer, viral and bacterial infections and for selecting vaccine candidates against various diseases (reviewed in [193–195]).

5.2.2. Protein profiling with antibodies

Antibodies, being uniform, relatively stable binders with a high affinity for target molecules, are the best candidates to assess the abundance of proteins in complex biological mixtures by measuring the relative number of captured molecules [7,120,186,196–201]. Three array strategies have been proposed to compare the expression of proteins by the evaluation of their interactions with monoclonal antibodies (Fig. 4).

A two-color approach detects the fluorescence intensity of spots by comparing two samples mixed in a 1:1 ratio, in which the same proteins labeled by two fluorophores of different wavelengths are in competition to bind to the corresponding arrayed antibodies [197]. Running two parallel experiments with mutually exchanged fluorophores used for labeling excludes the effect of the bioconjugation bias to proteins on the interpretation of the results. The relative protein concentration is measured by comparing the ratio of the fluorescence intensity of sample spots to the ratio of the intensity monitored for a reference protein. This approach has been applied to study protein profiling in pathological tissues and cells [196,198,202,203]. However, the two-color detection capacity is within about 3 log of concentrations and is limited in the assessment of low-abundance proteins in a single assay [186]. The two-color approach has recently been strengthened by coupling it to rolling-circle amplification, which increased by up to 30-fold the sensitivity of the detection of low-abundance proteins with a high accuracy and reproducibility in the 24 sera tested in parallel assays [204].

Another mono-color approach, called “competitive displacement”, detects protein abundance with antibodies immobilized in hydrogel through competition of target proteins in two samples mixed in increasing ratios, 1:1, 1:10, etc., when only one sample, considered as a reference, is labeled [205]. According to this approach, the proteins of similar concentrations in samples mixed in the ratios 1:1 and 1:10 would give, respectively, 50 and 90% displacement, which can be detected by the reduction in fluorescence intensity. For up-regulated proteins, the displacement level will be more significant whereas for down-regulated proteins it will be less significant. This approach moves away from the saturation of spots since the signal falls as a result of the antibody binding to non-labeled proteins therefore a wider range of protein concentrations might be detected in a single assay. However, it does not take into consideration that the conjugated fluorophores affect protein topology, molecular mass, solubility and diffusion in hydrogel thus labeled and non-labeled proteins can be rather different in their competitiveness, which might distort the profiling of proteins.

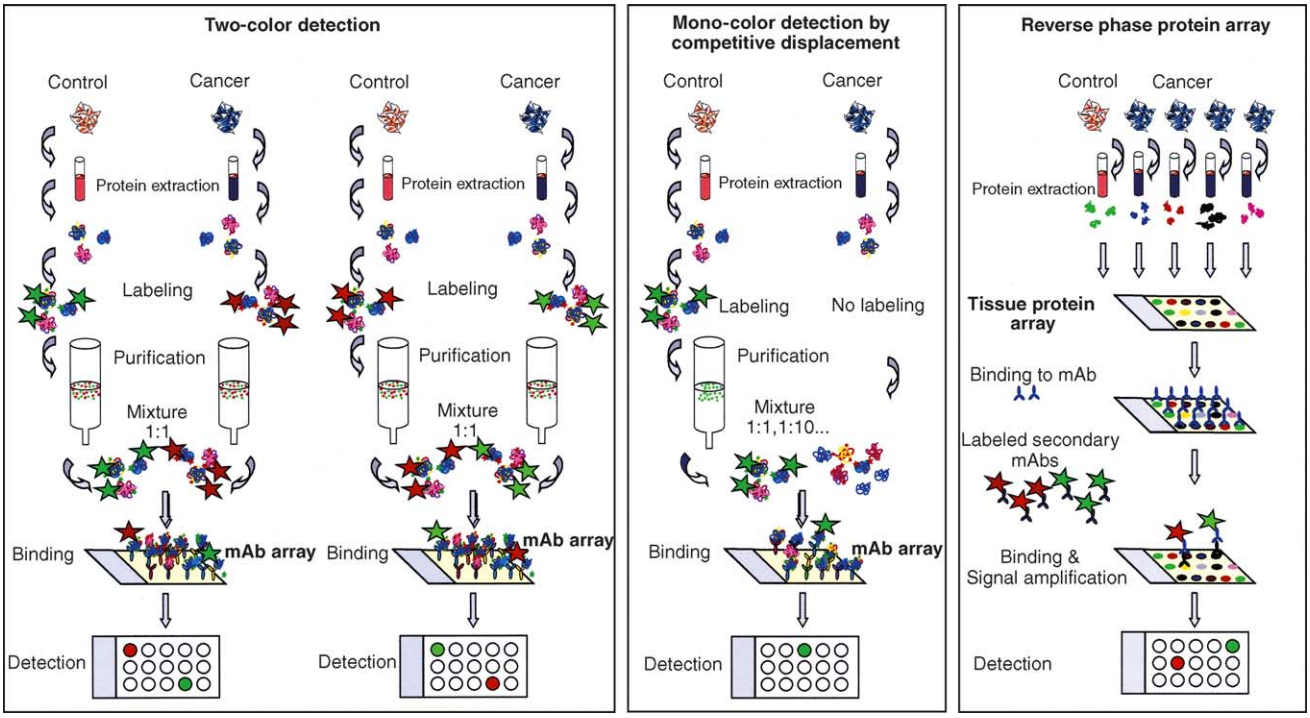

Fig. 4. Strategies for protein expression profiling by protein arrays. The “two-color” and “competitive displacement” approaches use antibody arrays whereas the “reverse phase” approach uses arrays of immobilized total proteins from tissue specimens. For details, see text.

In the third approach, referred to as "reverse-phase protein microarray", the positioning of antibodies and proteins is inversed in the experimental design [110,111,206]. It is the mAb that detects a target protein in a total protein fraction extracted from a biological sample and arrayed on the support. Such a direct capture assay to measure the protein concentration by chemiluminescence or fluorescence, including the detection of low-abundance proteins by the tyramide amplification method, is well suited to compare protein profiles in numerous clinical specimens [207].

A major hindrance to the performance of protein profiling approaches is the lack of a real specificity of available antibodies; only between 5 and 20% of commercial mAbs can be considered useful for protein profiling experiments [186,208]. Indeed, if a total cross-reaction and non-specific interaction strength prevails over the expected specific interaction with a target protein, then a relative measurement of captured proteins becomes impossible on spots. A traditional way to override this obstacle is the experimental design with two mAbs which recognize different epitopes in the same protein; the first to capture the protein and the second to select the captured protein. Such a sandwich method allows profiling to be assessed more precisely. It has been successfully applied to follow the synthesis and secretion of a limited number of proteins, such as cytokines, a group of mediator proteins involved in cell communication [120,199,200,209].

Recently, the proteome microarray has been proposed as a means to estimate the quality of antibodies, in terms of their cross-reactions with other proteins, and to choose the best candidates [210]. Furthermore, full-length antibodies can be replaced by smaller, more stable, soluble domain antibodies [211], by recombinant single chain Fv fragments (scFvs) [212], by antibodies from camel [213] which will appear to respond better to protein array technological requirements. Nucleic acid-based binders, like aptamers and photoaptamers, have also been tested as arrays for multiplexed measurement of proteins in serum samples [214,215]. The development of a new format of a double chip protein array, using two congruent surfaces that contain spotted protein and scFvs of interest, can also significantly diminish cross-reactions [216,217].

5.2.3. Protein domain arrays

Protein interactions are basically determined by the structural features of proteins. Two proteins can establish functional contacts through specific recognition of a short target sequence in one partner by a specialized interaction domain located in another partner [177]. A modular organization of regulatory proteins is also crucial for the molecular recognition of nucleic acids, phospholipids and small ligands. Moreover, it turns out that the cell uses a limited set of interaction domains, which, being able to fold independently and be presented in different combinations in proteins, can recognize various motifs in interacting partners. Therefore, they can direct distinct regulatory pathways such as phosphorylation-dependent and -independent signal transduction, ubiquitination, targeted proteolysis, cell polarization, endocytosis, etc. [218]. Interaction domains, usually comprised of 35–200 amino acids [219] and a short ligand motif can be predicted in suspected proteins by the identification of conserved amino acid sequences [220,221] and protein–protein interactions can be characterized experimentally using probes of degenerate synthetic or phage displayed peptides, yeast two-hybrid and mass-spectroscopic techniques [222]. The protein interacting domains described are grouped into protein families (Pfam), which are available in a database (http://pfam.wust.edu/).

The first protein domain microarray was prepared by immobilization of 212 purified GST-tagged proteins on a nitrocellulose membrane and probed to peptides potentially able to recognize 10 different protein interaction domains [223]. The detection of bound molecules was carried out with Cy3- and Cy5-labeled peptide probes or with anti-domain primary antibodies, which were recognized by appropriate FITS-conjugated secondary antibodies. Two-color detection of unmethylated and methylated arginine in a peptide carrying the P3 motif of Sam68 discriminated between proteins with SH3 domains that are highly sensitive to arginine methylation and less specific WW domains. The method was rather sensitive for detecting the characteristic binding profiles of endogenous proteins in total cellular extracts using primary antibodies against the dominant P3 and PGM interaction motifs located within proline-rich Sam68 and SmB′ regions, respectively. The acquired information has been useful in searching for low-molecular inhibitors of arginine methylation of *N*-methyltransferase involved in transcriptional activation [224].

Protein microarrays have also been used to identify interacting partners with tuberin, encoded by the tuberosis sclerosis complex 2 tumor suppressor gene [225]. Tuberin is a target for phosphorylation by Akt kinase that negatively regulates downstream signaling pathways and shares similar phosphorylation sites with other proteins like 14-3-3. Ten putative phosphorylation sites were predicted on tuberin in silico. A protein domain microarray was prepared for candidate domains and motifs including 14-3-3 proteins and screened for the ability to interact with phosphorylated and non-phosphorylated tuberin-specific peptides.

A coiled-coil domain (leucine-zipper that forms a globular structure) microarray was prepared from 49 highly purified peptides representing 16 families of human bZIP and 10 yeast proteins [226]. To exclude homodimerization, peptides were denatured, printed and probed with denatured fluorescent peptides. The strength of interaction in a range of 50 nM–3 μM could be measured using internal standards and binding was shown to occur with high selectivity although the bZIP coiled coils share remarkable sequence similarity. Peptides within similar families possessed close interaction strength, whereas peptides from different families formed distinct binding patterns. Furthermore, several new candidate interactions were identified that appear to be involved in intracellular signaling and circadian machinery-related pathways.

The WW domain, with two highly conserved tryptophan (W) residues spaced within a 30–40 amino acid stretch that binds proline-rich ligands, has been found in signaling proteins involved in cell-cycle control and co-activation of transcription [227,228]. Several subgroups of WW domains displaying different binding specificity with respect to WW peptide ligands have been described [229]. The WW domain is implicated directly or indirectly in muscular dystrophy, cancer, Alzheimer's and Huntington's diseases [230]. The array approach has been applied to map WW domain protein interactions using 96-well plates by a quantitative ELISA-like binding assay [221]. A total of 2189 putative WW peptide ligands, with a length ranging from 10 to 16 amino acids, were probed to 30 WW GST-fusion domains. The WW functional domains were classified by their ability to bind four subgroups of peptide ligands. The construction of the first WW domain–ligand protein–protein interaction map by a protein array is of particular importance in a better understanding of severe pathologies and in the search for new therapeutic targets and WW domain specific inhibitors.

The fact that interaction domains alone, being isolated from the context of corresponding proteins as small and globular structures, are still able to fold correctly and interact specifically gives priority to the array method over other high-throughput methods in studying protein–protein interactions. Further application of protein domain arrays can accelerate the characterization of functional interaction maps in organisms and will, therefore, have a greater biomedical importance.

6. Conclusion

The specificity of protein interactions takes on special significance as a general problem in biology. Protein interactions with other proteins and with DNA by specialized binding modules determine primarily the diversity of biological processes and orchestrate the fundamental regulatory and signal pathways in organisms. Physically interacting proteins appear to be precisely co-expressed to preserve the proper stoichiometry among partners and perhaps have evolved in a coordinated manner to conserve functionally important contacts [231]. The elucidation of functionally significant interactions on a large scale might help to understand both the role of individual interacting partners and the order of cascade protein interrelations in cellular regulatory and signaling pathways as well as the consequences of an interruption of a given interaction in the development of pathological processes.

The technological advances in the use of protein arrays mark a new step in proteomics by providing a high-throughput and multiplexed method to study protein interactions for basic and applied purposes. It appears that the coupling of genomics with proteomics through the fabrication of defined protein arrays based on the knowledge of genome sequences will be the quickest and most efficient way to understand structure–function relationships in entire organisms. Protein arrays address the expression profile and post-translation modifications of proteins, the assessment of the immune response, the identification of biomarkers, the screening of targets for therapeutic purposes, the choice of vaccine candidates and the search for new leads. Obviously, various formats of protein arrays will become indispensable to solve urgent biomedical problems.

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