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Developing Prospects of Microarray Proteomics

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Abstract

"Proteomics" can be defined as a field of research studying proteins in terms of their function, expression, structure, modification and their interaction in physiological and in pathological states. It is not only a powerful research tools for the proteomics, but also a new means of early diagnosis, prognosis and treatment evaluation in the clinical application. Protein microarray technology possesses some of the greatest potential for providing direct information on protein function and potential drug targets. In the near future, protein chips may allow construction of complete relational databases for metabolic and signal transduction pathways. For example, functional protein microarrays are ideal tools suited for the mapping of biological pathways. The principle, classification, preparation, application, advantages, disadvantages and development prospects of the Microarray Proteomics are introduced. Protein microarray technology possesses some of the greatest potential for providing direct information on protein function and potential drug targets. We strongly believe that functional protein microarrays will soon become an indispensible and invaluable tool in proteomics research and systems biology.

Keywords: Microarray proteomics; Protein chip technology; Progression

Introduction

High throughput analysis as the DNA microarrays provides a single DNA microarray studies on the ability of the genome. Time-consuming and complex analysis can be simplified, and the processing time can be shortened to a few minutes. In contrast to genomic approaches like DNA or RNA microarrays, proteome-based microarrays have the ability to measure changes in the proteome directly. Protein microarrays are able to give insight into protein abundance and composition after post-translational modifications, splicing events, poly-morphisms, localization, and interactions of all proteins in one sample [1]. Nevertheless, determining the protein expression of cells and tissues is a challenging task on the way to characterize the proteome. Those different techniques and methods could display the wide range of protein concentrations and their stoichiometry to each other. Two ways, relative and absolute quantification provide additional information. Quantifying proteins in an absolute manner gives exact information about total (or specific) protein content of a sample whereas, relative quantification is able to show relationships between proteins, protein families and complexes. Therefore, the knowledge about the protein composition may help predict disease progress, perform risk stratification and identify pathogenic mechanisms [2]. Frequently, changes in the human proteome can be linked directly to specific clinical phenotypes. Systematic analysis of biological processes needs to understand the quantitative expression pattern of proteins and their interaction partners. The necessity to detect even small changes in the wide diversity of proteins in response to an altered state demands accurate quantification approaches. Thus, the employment of these techniques involves large amounts of cellular material which is a major drawback for systems working with limited amounts of primary material like patient samples or stem cells. Moreover, Mass spectrometry (MS) analysis requires the reduction of sample complexity by immunodepletion, fractionation or enrichment techniques, whereas a risk of protein co-depletion still remains. Protein microarrays could be a promising alternative to complement and supplement MS techniques [3]. Currently, comprehensive applications resulted in a broad spectrum of protein micro-arrays (Figure 1).

Classification of microarrays proteomics

Proteomic microarrays are usually divided into two categories: a functional protein microarrays and protein microarray detection [4]. The immobilization of different purified proteins, protein domains or functional peptides are elements of protein function microarrays. These types are usually used for microarray screening of molecular interactions and potential interaction partners. On the other hand, protein microarrays are specific protein capture agent which can specifically recognizes specific proteins from a complex mixture. These chips can be used for protein profiling, for example: quantitative and abundance proteins in complex mixtures posttranslational modification evaluation.

Functional microarrays proteomics

Understanding of the molecular interaction networks, which defines a specific group of proteins is one of the main goals of functional proteomics. Functional proteomics microarray provides a very powerful tool to accomplish this daunting task, especially when assessing the activity of families of related proteins. In 2000, Schreiber et al. showed that purified recombinant proteins could be microarrayed onto chemically derivatized glass slides without seriously affecting their molecular and functional integrity [5]. Snyder et al. have been able to make about 5,800 on the baker's yeast proteins fixed to microscope glass slides [6]. This protein chip, then probing with different phospholipids to determine several lipid-binding proteins. The same authors also used the protein chip to identify 87 different protein kinases carrier [7]. By using this microarray data set in combination with protein-protein interaction and transcription factor

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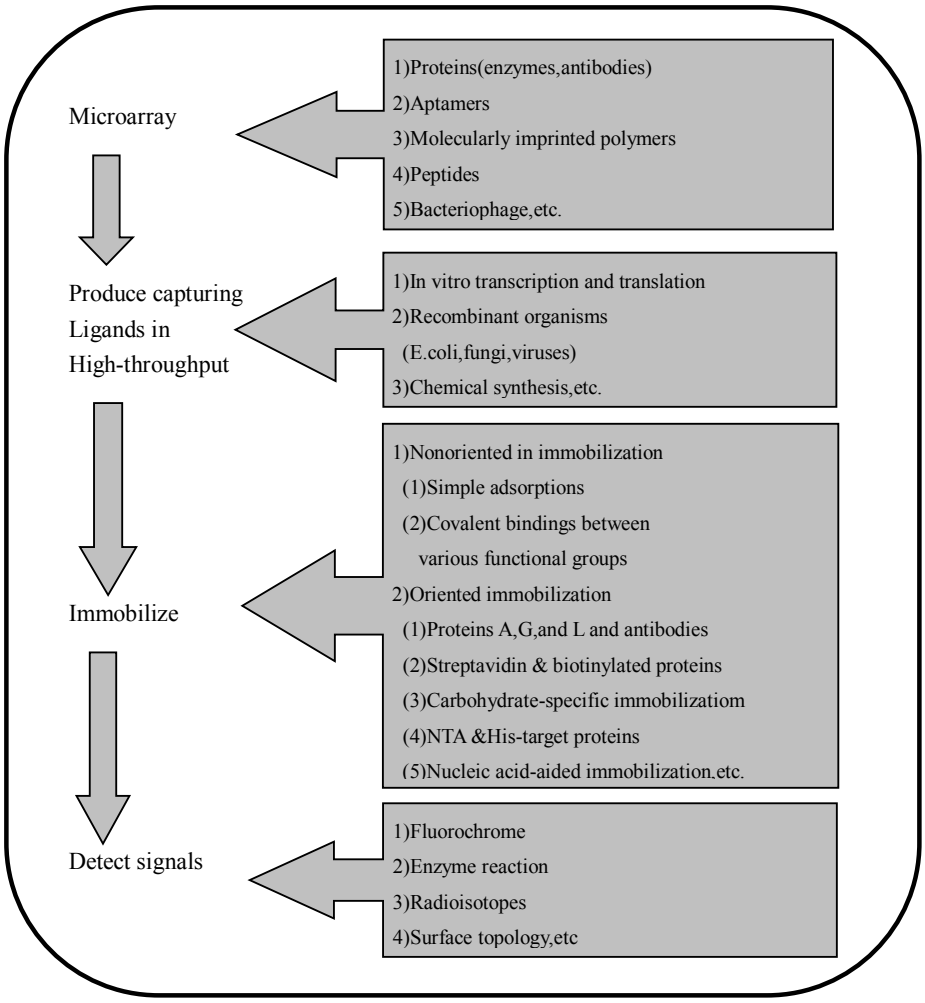

Figure 1: Schematic representation for strategy fabricating protein microarrays.

binding data, the authors were able to reveal several novel regulatory modules in yeast [7]. By using a similar approach, Dinesh - Kumar and his colleagues constructed a protein microarray containing 2,158 unique Arabidopsis thaliana proteins. This array is used to identify 570 mitogen-activated protein kinase phosphorylation substrate. These mitogen-activated protein kinase phosphorylation substrate including regulation included several transcription factors involved in the regulation of development, host immune defense, and stress responses [8]. The analysis of proteome-wide microarrays from yeast was also recently used to find unexpected non-chromatin substrates for the essential nucleosomal acetyl transferase of H4 (NuA4) complex [9]. In this interesting work, the authors discovered that NuA4 is a natural substrate for the metabolic enzyme phosphoenolpyruvate carboxy kinase and that its acetylation is a critical regulator of yeast chronological lifespan [9]. In another example, human proteome arrays were used for the detection of autoimmune response markers in several human cancers [10]. Kirschner et al. also used the human proteome arrays to identify novel anaphase-promoting complex substrates [11]. This was accomplished by probing the arrays with cell extracts that replicate the mitotic checkpoint and anaphase release and then probing the captured proteins with antibodies specific for detecting poly-ubiquitination [11]. Functional protein micro-arrays have also been used to study families of interacting protein domains. Bedford and his colleagues have shown that some protein domains (FF, FHA, PH, PDZ, SH2, SH3, and WW) may be fixed in the micro-array format, retaining their ability to mediate specific interaction [12].

Functional protein domain microarrays can also be used to determine protein interactions. For example, Black and colleagues used microarrays containing multiple variants of the transcription factor p53 to study and quantify their DNA-binding preferences [13]. The use of fluorescent-labeled DNA probes can produce binding isotherms and extract the different equilibrium dissociation constants for each p53 variant [13]. Mac Beath et al. have also used a similar method to determine the interactions of several human SH2 and PTB domains with different phosphotyrosine-containing peptides derived from human ErbB receptors [14]. Furthermore, they have the potential to generate data that, when collected in a quantitative way, could be used for training predictive models of molecular recognition [15,16]. Mac Beath and his colleagues recently used functions microarrays which contains multiple murine PDZ protein domains to screen potential interactions with 217 genome-encoded peptides derived from the murine proteome [15,16]. The data generated was used to train a multidomain selectivity model to predict PDZ domain-peptide interactions across the mouse proteome. Interestingly, the model indicated that PDZ domains are not separated into discrete functional class; on the contrary they are

uniformly distributed in the spatial selectivity. This finding strongly suggests that the PDZ domains across the proteome are optimized to minimize cross-reactivity [15,16].

Protein-detecting microarrays

As described above, functional protein microarrays allow high-throughput screening and quantification of protein interactions on a proteome-wide scale, thereby providing an unbiased perspective on the connectivity of the different protein-protein interaction networks. Establishing how this information flows through these interacting networks, however, needs to measure the abundance and post-translational modifications of many proteins from complex biological mixtures. Protein-detecting microarrays are ideal reagents for this type of analysis. One of the most frequently used strategies to prepare this type of microarray includes the use of monoclonal antibodies as the capture reagent of specific proteins. Antibodies have been classically well suited for this task, because a large number of specific antibodies are commercially available, which can be easily immobilized on a solid support [17,18]. The potential problems associated with the use of antibodies for chip assembly might manifest through moderate expression yields and by issues related to the stability and solubility of these large proteins. These potential problems have led to the exploration of alternative protein scaffolds as a source for new, more effective and stable protein capture reagents [19]. Suitable protein scaffolds that have been proposed include the Z domain of protein A, fibronectin domains, lipocalins and cyclotides, among others.

Generally, the antibody microarrays are ideal for detection of protein abundance the biological sample having a relatively large dynamic range [20]. For example, Haab made use of antibody microarrays for serum-protein profiling in order to identify potential biomarkers in prostate cancer [20]. Using this approach, the authors were able to identify five proteins (immunoglobulins G and M, α1-anti-chymotrypsin, villin and the Von Willebrand factor). They had significantly different levels of expression between the prostate cancer samples and control samples from healthy individuals.

In a similar fashion to that of a sandwich ELISA assay, antibody microarrays make use of a second antibody directed towards a different epitope of the protein. This contributes to the detection and quantification of the corresponding analyte, facilitates the detection and quantification of the corresponding analyte. This approach has been used for monitoring changes in the phosphorylation state of host proteins [21], including receptor tyrosine kinases [22], and for serum protein profiling to identify new biomarkers in prostate cancer [23] among other applications. Using this method is generally limited by the availability of suitable antibodies that can be used for capture and detection. Furthermore, the detection step requires the use of multiple fluorescent labeled antibody, which may increase the background signal and the risk of cross-reactivity with the number of antibodies increases. One way of overcoming this problem is to use one or more fluorescent dye labeled the protein of biological sample [24]. This approach allows one to perform ratiometric comparisons between different samples by using spectrally distinct fluorophores and has been used to detect molecular biomarkers of different types of human cancer [25,26]. It should be emphasized; however, non-specific chemical marker protein is introduced into chemically modified on their surface. Thereby changing the antibody recognizes and causes false signals. However, nearly all of the different methods available for this task (see below) still lack the sensitivity required for most biological applications These weaknesses can, in principle, be avoided by using a label-free detection scheme. However, almost all of the different methods available for this task also lack the sensitivity needed most biological applications.

Although antibody microarrays are well suited for protein profiling, proteome-wide applications have not been accomplished. This is mainly due to the lack of effective, well-validated antibodies. An ingenious solution presented by Lauffenburger et al., however, is to use a combination of different experimental methods and data generated by microarray [27,28]. In this work, the authors combined data, which gathered from antibody microarrays, enzymatic assays, immunoblotting, and flow cytometry, to assemble a network of almost 10,000 interactions in HT-29 cells treated with different combinations of cytokines [27]. All of this information was later used to uncover mechanisms of crosstalk involving pro- and anti-apoptotic signals induced by different cytokines [28].

Protein lysate microarrays

An interesting alternative to antibody microarrays is to immobilize cell lysates and then use specific monoclonal antibodies to identify and quantify the particular analyte in the corresponding lysate. This technology was first described by Liotta to monitor pro-survival checkpoint proteins as a function of cancer progression [29]. This approach has been used for the discovery and validation of specific biomarkers for disease diagnosis and patient stratification. Utz and his coworkers have made use of lysate microarrays to study the kinetics of intracellular signaling by tracking 62 phosphorylation sites in stimulated Jurkat cells [30]. They have discovered a previously unknown connection between T-cell receptor activation and Raf-1 activity [30].

Every spot in the protein lysate microarray contains the entire set of biological proteins to be analyzed. It is necessary to prepare as many copies of the array as proteins needed to be analyzed, in order to analyze the abundance and modification states of different proteins present in the lysate. Lysate microarrays also denature the proteins during the immobilization step onto the solid support. This makes it impossible to research complex protein-protein interactions and needs to use of specific and well-validated antibodies for the recognition of specific continuous protein epitopes. This is a serious limitation of this technique, since it only allows the analysis of proteins that have already been discovered and to which suitable antibodies are available. It should be noted that the majority of commercially available antibodies typically show substantial cross-reactivity issues and are not appropriate for this type of approach. Only antibodies can provide a single band in a standard Western blot should be used. Furthermore, blocking and detection protocols, and the composition of the lysis buffer has been shown to significantly affect the performance of the antibody [31], thus indicating that further developments needs to widespread use of this technology.

Advantages and Limitations

In recent years, protein microarray technology further improved on the way to quantitative proteomics. In order to ensure uniformly high quality microarray data, different technical aspects are under consideration. High quality data can be gathered if the technical variability is less than the investigated biological variability.

One aspect is the availability of high quality affinity reagents and rigorous antibody validation, which gained wide acceptance. Previous studies have revealed that the antibody reactivities on protein microarrays differ from those yielded by Western blotting, although the same antibodies and lysates under identical assay conditions were tested [32]. They concluded that the conditions using in WB and

protein microarrays are not identical. By using WB, the off-target proteins are separated from the proteins of interest before antibody detection. Thus, protein microarrays are more susceptible to antibody cross-reactivity. Moreover, protein microarrays are often applied to study changes in signaling pathways. The detection of such dynamical changes that depends on various factors is a challenging task for protein quantification. The performance of each antibody can vary, depending on cell type, treatment of the cells and time points that are investigated. These results suggest that antibodies used in protein microarrays should be evaluated not only in WB, but also under microarray conditions, as well as screened with more cell types and conditions. On the basis of these, Sevecka et al. [33] suggested a two-stage procedure. They screened a library of 383 antibodies. These antibodies are screened against 20 biological con-texts, each of them representing a specific combination of cellular background and treatment condition and across 17 different cell lines. Candidate antibodies that showed significant difference in signal intensities were further evaluated by WB.

This method has the potential to standardize antibody validation. Therefore, the authors suggested that the proposed scheme should be adapted to other biological context and applied to other high throughput immunoaffinity assays. Sjoberg et al. [34] introduced a standardized protein array protocol for the verification of affinity reagents from different sources. Nevertheless, there is lack of a general accepted standard to address antibody specificity and selectivity. It is determined by the views of researchers, whether an affinity reagent is specific or not. A significant effort is the generation of large libraries of fully characterized specific antibodies and other affinity reagents. Several international consortia like the 'Human Proteome Atlas' [34] and Alliance for Cellular Signaling [35] focus on the development of well validated antibodies. A dual-color read-out, in which the target proteins are directly labeled, reduces the number of specific antibodies needed for the assay and allowing the analysis of two samples in one experiment [36]. The major defect of this method is the homogenous labeling of proteins across all samples in one study. Furthermore, the nature of non-specific chemical labeling of proteins involves the problem that they may be labeled preferentially on reactive amino acids that may alter antibody recognition and can lead to false positive/false negative signals.

With respect to the diversity of microarrays, a multitude of different factors are crucial for the quality and reliability of data. Furthermore, protein and peptide quality vary from batch to batch. In addition, the low level of consistency between different commercial protein array platforms [37] and different normalization strategies, exemplarily shown for peptide microarrays by Hecker et al. [38], underline the need for commons standards. Therefore, protein researches require individual standards for every type of protein microarray and their applications. It should be the aim to develop standard protocols for protein and peptide microarrays, similar to the MIAME checklist for DNA microarrays [39] or Mini-mum Information about a Proteomics Experiment providing guidelines for the standardization of proteomic data derived from MS [40]. Although, these standards exist and often are an inherent part of journal guidelines, Witwer reviewed that about one quarter of microarray-based microRNA studies out of 120 articles were not fully compliant with the reporting standards [41]. MS-based proteomics is now the only method used for the systemic characterization of proteins from identification, quantification, and characterization of either post-translational modifications or protein interactions [42].

Microarray Application

In the last decade alone, microarrays have evolved from being used primarily as basic analytical research tools to now viable options for more sophisticated applications in proteomics, including protein expression profiling, molecular interaction mapping, biomarker and drug discovery, disease diagnosis, and vaccine development. In this section, we will elaborate on recent studies of microarray applications that focus primarily on three areas most relevant to proteomic research, namely functional annotation, substrate fingerprinting, and ligand/inhibitor binding, with each application taking full advantage of a microarray's key features: miniaturization and parallelization.

Functional annotation

With conventional protein screening assays, the functional annotation of proteins is usually performed by incubating them with appropriate substrates, which will report protein activities in the form of absorbance, fluorescence, or luminescence signals. About ten years ago, the first microarray-based strategy for rapid and reliable functional annotation of proteins was developed [43]. The strategy uses fluorescently labeled activity-based probes, which detect corresponding enzymes based on their intrinsic enzymatic activity by the formation of covalent probe-enzyme complexes. In a proof-of-concept experiment, a total of 12 proteins were immobilized onto epoxy-functionalized slides and screened with a panel of different activity-based probes. The results provided clear evidence that the proteins were successfully detected on the basis of their enzymatic activity. At the next stage, this strategy was extended to profile proteases with a panel of activity-based probes by virtue of enzymatic activities and substrate specificities [44,45]. These reports laid the groundwork for potential high-throughput screening of enzymatic activities and inhibition in a protein microarray. Eppinger used the same strategy to quantitatively determine enzyme kinetics on a microarray [46]. Researchers were able to obtain kinetic information of the enzyme directly from the resulting microarray data, by immobilizing papain on hydrogel slides and incubating it with a fluorescently labeled suicide inhibitor. The strategy was extended to the research of six cathepsins against seven inhibitors to obtain the corresponding inhibition constants that were later shown to be consistent with previously reported data [47]. Jung and his coworkers have developed a new surface-concentration-based assay for quantitative kinetic analysis of proteases on microarray [48]. In this method, a series of peptides with rhodamine were immobilized onto maleimide-funtionalized slides to derive quantitative kinetic data such as Michaelis constant and maximum velocity using dry-off measurements. Cravatt and colleagues took an alternative approach to design a novel microarray platform that enables proteomic profiling of enzyme activities, by integrating an activity-based probe and an antibody microarray [49]; a proteome was first incubated with probes. The labeled enzymes were then captured and anchored onto the antibody microarray to identify the specific enzyme. Compared with traditional gel-based methods, the array-based method minimized the consumption of expensive reagents and improved the sensitivity to detect enzymes. High quality antibodies were required for the successful implementation of this approach

The application of microarray technology has been extended to the research of functional protein pathways. Lackner et al. developed a reverse-phase protein array to analyze the phosphorylation status of 100 proteins with different breast cancer cell lines [50]. Cellular lysates from various cell lines were spotted onto the slide in serial dilutions and probed with different antibodies that recognize phosphorylated

proteins. The study allowed the researchers to perform signaling pathway network analysis and classify breast cancer cell lines into different subtypes. Furthermore, microarray analysis can also yield valuable information on the deregulated signaling pathway in individual cancers.

Substrate fingerprinting

One of the main applications of microarray in proteomics is to map ligand binding specificities of a protein, which is essential to understand the role and interactions of protein's physiological. Especially for the enzyme, the substrate specificity related information is crucial for a better understanding of their many cellular functions. A comprehensive knowledge of enzyme substrate specificity can help the successful design of highly potent and selective inhibitors, facilitating the drug-discovery process.

Epigenetic modifications can have a profound influence on a variety of human diseases. Mrksich et al. synthesized a peptide library to research the substrate specificities of different lysine deacetylases [51]. Arrowsmith and his coworkers constructed a position-scanning peptide library on cellulose membrane based on two histone peptides, histone 3 trimethyl lysine 9 and histone 3 trimethyl lysine 27, to profile the substrate specificities of chromo domains [52]. Knapp et al. synthesized a library of peptides containing all acetylated lysine sites from histone proteins on cellulose membranes and investigated the binding preferences of 43 different bromo domains [53]. This research led to the identification of 485 new protein-histone interactions, a number of which were further confirmed by isothermal titration calorimetry. The research revealed that posttranslational modifications could exert significant influence on peptide/ bromo domain Interactions.

Research on substrate specificity of kinases is another area of active investigation that has been ongoing since the early 2000s. In a research, a microarray that contains 290 Tyr peptides and 1,100 Ser/ Thr peptides, was constructed and used to investigate the substrate specificity of several kinases [54]. By this method, the researchers not only confirmed previously identified kinase-recognizing motifs but also uncovered many new sequences with high potency and selectivity. This high-density peptide array approach can provide a powerful tool to facilitate the discovery of potential substrates of other kinases in a high-throughput and sensitive manner.

Previous phosphopeptide microarrays had primarily focused on profiling enzymatic activities of different protein phosphatases [55]. In this new study, high-affinity, selective peptides designed for individual SH2 domains were first identified from microarray and then examined by pull-down experiments. It was found that peptides identified from the peptide microarray could successfully pull down target proteins directly from crude cellular lysates. Further pull-down experiments with these peptide hits conducted the identification of three potential cancer biomarkers, highlighting the feasibility of this microarray strategy to facilitate future biomarker discovery.

Computational prediction methods have been integrated into peptide array for proteome-wide profiling of substrate specificity of proteins. Denu et al. utilized SPOT array to screen SIRT3 binders from both known and potential peptide substrates [56]. Based on the array results, they developed a machine-learning method to establish binding trends and predict new binding sequences from the mitochondria proteome. Results from this experiment indicated that SIRT3s are involved in several metabolic pathways and new enzyme/substrate interactions could be discovered. In 2012, Wang et al. combined computer modeling and bioinformatics analysis to filter around 700 potential binders of the Abl1 SH3 domain [57]. The study indicated for the first time that the Abl1 SH3 domain may interact with numerous methyl transferases and RNA-splicing proteins. Through domain peptide recognition events, this strategy may offer a practical pathway to detect novel protein interactions.

Ligand/Inhibitor Binding

Small molecule microarrays are robust tools to identify potential proteins binders. According to standard operational procedures, proteins can be labeled with a fluorescent dye and then incubated with an array of small molecules. The detected fluorescent intensity can be used as a guide to identify the small molecule binders of the proteins. The reference protein can be screened within the same platform, if necessary, to check for cross-activity. From such results, highly specific and strong small molecule binders of a target protein could be rapidly identified. Notably, the random dye-labeling methods may interfere with protein function and sometimes can even lead to the denaturation of proteins.

Schreiber and his colleagues were among the first teams to develop small molecule microarrays for ligand discovery. With their diversity-oriented synthesis strategy strategy, several small molecule microarrays were successfully fabricated in the early 2000s. For example, a small molecule microarray with 18,000 compounds was constructed and screened against the protein calmodulin for potential binders [58]. A compound identified could induce cardiovascular malfunction in a zebra fish phenotypic assay. The team has anchored over 15,000 small molecules from a variety of sources to an array and studied the binding affinity of 100 various proteins [59]. It was found that increasing the content of sp3-hybridized and stereogenic atoms in the compound library generally improves the protein-binding selectivity of library members. This finding could obtain helpful information in the future design of compound collections with improved biological activities. By integrating small molecule and peptide hybrid libraries and microarray technology, Wu, H and his coworkers took a different approach to uncover small molecule inhibitors of 14-3-3σ [60]. A 243-member N-terminal library and a 50-member C-terminal hybrid library were synthesized and anchored onto the array to screen for potent binders of 14-3-3σ.

Summary

Inaugurated as an analytical tool for proteomics research, the various forms of microarray technologies, including protein microarray, peptide microarray, and small molecule microarray, have gradually evolved into robust platforms to facilitate drug discovery and diagnostic applications. With the progressive development and more innovative breakthroughs in the foreseeable future, microarray technology promises to elevate its scope of research and potential applications to a higher level. We strongly believe that the protein microarray technology is on the brink of becoming a standard technique in research in the same way as DNA microarray technology is used today.

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