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Review

Recent Advances in Microarray Technologies for Proteomics

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Proteins are fundamental components of all living systems and critical drivers of biological functions. The large-scale study of proteins, their structures and functions, is defined as proteomics. This systems-wide analysis leads to a more comprehensive view of the intricate signaling transduction pathways that proteins engage in and improves the overall understanding of the complex processes supporting the living systems. Over the last two decades, the development of high-throughput analytical tools, such as microarray technologies, capable of rapidly analyzing thousands of protein-functioning and protein-interacting events, has fueled the growth of this important field. Herein, we review the most recent advancements in microarray technologies, with a special focus on peptide microarray, small molecule microarray, and protein microarray. These technologies have become prominent players in proteomics and have made significant changes to the landscape of life science and biomedical research. We will elaborate on their performance, advantages, challenges, and future directions.

It's the Proteins' World

The completion of the human genome project has revolutionized the way we think about various diseases, shifting our view to the gene level. However, in most cases, genes are involved in the modulation of cellular functions only indirectly, through products they code for. Thus, the gene products, proteins, which are involved in virtually every process in the complex and well-integrated signaling, metabolic, and other cellular networks, are chiefly responsible for controlling diverse cellular activities by interacting with their partners specifically and tightly. It is estimated that more than 100,000 proteins exist in the human proteome alone (Pandey and Mann, 2000). This number illustrates the breathtaking challenge facing the systems-level analysis of proteins, or proteomics, which aims at deconvoluting the functions of each protein and linking them to specific cellular events. Compounding the challenge is the fact that the majority of proteins in the human proteome are also targets of numerous posttranslational modifications (PTMs), adding another level of complexity in the protein-interaction network and the modulation of protein functions (Pflieger et al., 2011). PTMs can significantly alter the basic properties and functions of proteins and consequently determine the fate of a cell, triggering one pathway versus another and regulating life/death decisions that a cell needs to make. It is widely known that deregulation of proteins can lead to numerous diseases, including cancers, diabetes, and neurological disorders. This means that proteins represent a large number of validated drug targets. Thus, the design, discovery, and development of effective drugs that may specifically turn off functions of target proteins (Schreiber, 2011) have been of growing interest to both the pharmaceutical industry and academic laboratories in recent years. The core principle of drug design is governed by two important criteria: potency and selectivity. In an ideal scenario, the drug should bind to its target proteins with strong affinity and at the same time not introduce interference to other proteins. The quest for suitable compounds as drug candidates can be a daunting task with traditional screening methods. To identify a few biologically active compounds from the huge libraries of biomolecules is like searching for a needle in a haystack. Without more sophisticated screening methods, the process of drug discovery will continue to consume disproportionately huge amounts of resources and efforts.

The above-mentioned challenges and obstacles call for high-throughput screening tools that can significantly accelerate the drug-discovery process and allow large numbers of protein-interacting and protein-functioning events to be analyzed in a rapid and efficient manner. In the last two decades, microarray presented itself as a highly viable solution. With this powerful and robust biotechnology, thousands of distinct biological moieties, such as DNAs, peptides, small molecules, and even cells, can be arrayed on a single slide and screened simultaneously (Uttamchandani et al., 2006; Sun et al., 2006; Wu et al., 2011a; Foong et al., 2012). Compared to traditional screening platforms such as microplate-based methods, microarray technology offers several prominent advantages, including miniaturization and parallelization. Though the technology was first introduced as miniaturized DNA assemblies on chips (Schena et al., 1995), it was not long before further pioneering efforts made it possible to sequester small molecules, peptides, and proteins in addressable grids (MacBeath et al., 1999; MacBeath and Schreiber, 2000). Since then, we have witnessed flourishing development in microarray technology and the emergence of various microarray-based platforms, including peptide, small molecule, and protein microarrays, which are the subject of this review. Microarray technology has altered the scope of life science research and enhanced

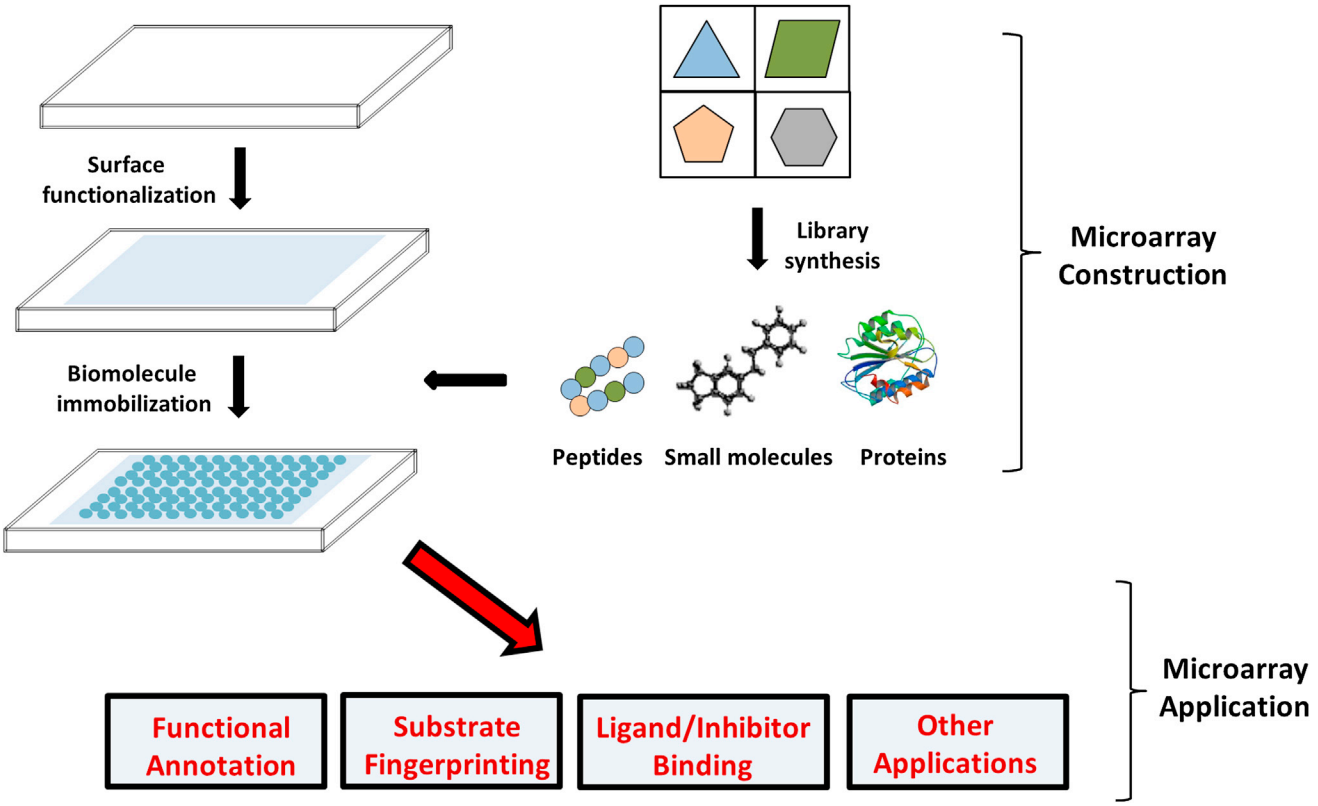

Figure 1. Overview of Key Microarray Technologies and Their Applications in the Field Of Proteomics

our perception and understanding of complex biological systems. Within the field of proteomics alone, microarrays have been successfully demonstrated for a variety of protein-related events, including studies of protein expression, interaction, function, and posttranslational modifications, some of which will be discussed in detail in the following sections. We will focus on some of the most noteworthy breakthroughs that have occurred in the last 2–3 years. All microarray screening technologies require several steps of operation that are essential to ensure a high level of performance. Most importantly, the success of each microarray-based screening heavily depends on the library construction and microarray fabrication. Therefore, we will begin by discussing the principles of library construction and microarray fabrication, providing separate overviews of various approaches currently employed in these leading platforms (e.g., peptide, small molecule, and protein microarrays). We will next discuss recent applications of these technologies pertaining to proteomics research, with a focus on three key areas of protein characterizations: functional annotation, substrate fingerprinting, and ligand/inhibitor binding (Figure 1). We will summarize some other recent applications of these platforms in proteomics. We will then conclude our review by offering our views on outstanding challenges as well as ways to further advance the microarray technology. It should be noted that other microarray-based technologies such as DNA, cell, and tissue microarrays have occasionally been used in proteomics, but they will not be the subject of the current review.

Library Construction

As a fundamental and crucial step in microarray screening, the construction of biomolecule libraries (henceforth referred to those of peptides, small molecules, and proteins) is also the most time-consuming phase in the entire microarray process. Substantial effort is required to ensure successful synthesis of the library.

Biomolecule libraries can be constructed in two ways. They can be directly synthesized in situ on the array, or they can be generated separately and then deposited onto the array. The earliest example of in situ synthesis was rooted in the form of SPOT synthesis (Frank, 1992), which provided a facile and convenient method to create peptide libraries on solid supports such as cellulose membranes. The method sometimes is referred to as the ''macroarray'' in order to draw a clear distinction from most of the microarray platforms to be discussed in this review. This membrane-based method does not require expensive automated instruments and can be performed expeditiously. In recent years, it has been extended to the synthesis of other types of synthetic molecules (Frei et al., 2012). The key advantage of in situ synthesis is the elimination of the spotting process. It should be noted, however, that the chemistry utilized for in situ synthesis should be clean and highly efficient because it will directly affect the quality of the microarray. In a recently reported strategy, Balakirev and coworkers constructed a surface-tension small molecule microarray in situ to screen the inhibitors of NS3/4A serine protease of hepatitis C virus (Mugherli et al., 2009). A library of 20,100

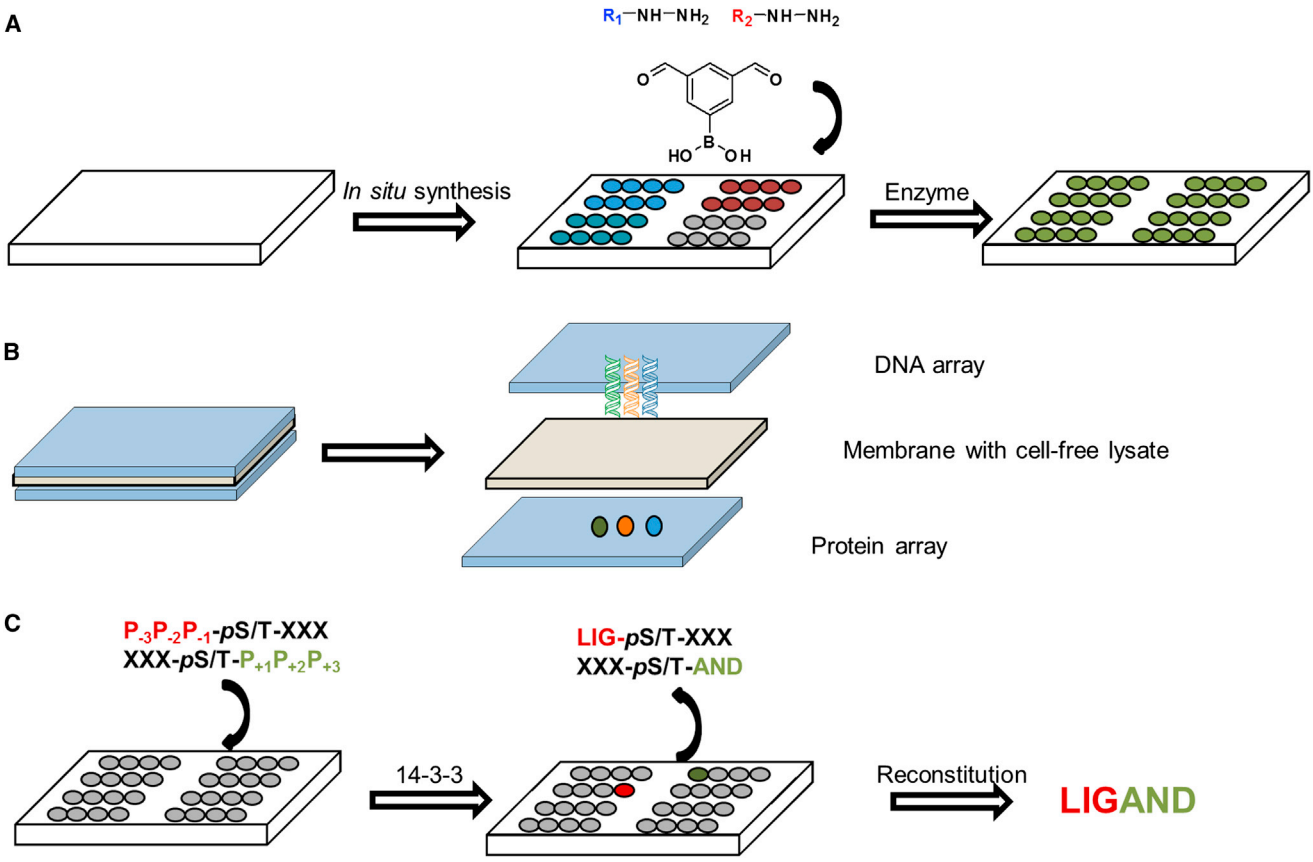

Figure 2. Representative Examples of Fabrication of Various Biomolecule Libraries
(A) In situ synthesis of the small molecule library on the microarray.
(B) In situ synthesis of the protein microarray through the use of DNA microarray.
(C) Synthesis of the peptide microarray by fragment-based approach.

dihydrazones was synthesized in situ through the reaction between 200 hydrazides and an aldehyde-containing boronic acid (Figure 2A). This library was then diluted with DMSO, and a solution of protease and fluorogenic substrate was applied to the library via a piezoelectric dispenser to examine the inhibitory activities. A number of compounds with low micromolar IC_{50} were discovered through this approach. For example, compound 201: 201(**1**; see Figure 3) has been identified with a low IC_{50} value of 1.5 μM. More recently, the same group has identified new fluorophores with drug-like property using multicomponent reactions performed in droplet arrays (Burchak et al., 2011). A total of 1,600 compounds were synthesized directly on the array by mixing eight heterocyclic amidines, 40 aldehydes, and five isocyanides. Several scaffolds were identified with fluorescence, and one of the compounds was found to stain a benzodiazepine receptor in bioimaging experiments. In 2008, He and coworkers described a new approach to convert DNA arrays to protein arrays in situ (He et al., 2008). In this approach, cell-free protein synthesis was conducted with a novel design using a membrane filter sandwiched by two slides (Figure 2B). Proteins were first synthesized on one slide, which was arrayed with DNA templates, and then diffused through the membrane filter and captured on the other slide coated with capturing agents. This approach has been applied to the fabrication of protein microarrays containing a variety of proteins expressed through cell-free systems, including antibody fragments, GFP, and transcription factors.

The other method of synthesizing the biomolecule libraries first and then depositing them robotically onto the glass slide is clearly more tedious but significantly more robust, and it has been the main method for the construction of most microarrays. It also has the advantage that many microarray replicates can be conveniently fabricated. This helps to save both the cost and time needed in the subsequent screening process when large numbers of arrays are required. This approach also boasts a higher degree of miniaturization and is therefore more economical when large-scale synthesis is conducted. We shall look at how different types of biomolecules are constructed using this method in detail in the following paragraphs.

Peptide Libraries

Peptide libraries represent a very important source for microarray applications due to the well-established solid-phase peptide synthesis (SPPS; Merrifield, 1985) as well as the comparatively easy fabrication of the corresponding microarrays. The SPPS bypasses the tedious synthetic effort normally required for the preparation of synthetic compounds (e.g., small molecules). Since its invention in the 1960s, the method has now been extended to the synthesis of a variety of other compounds,

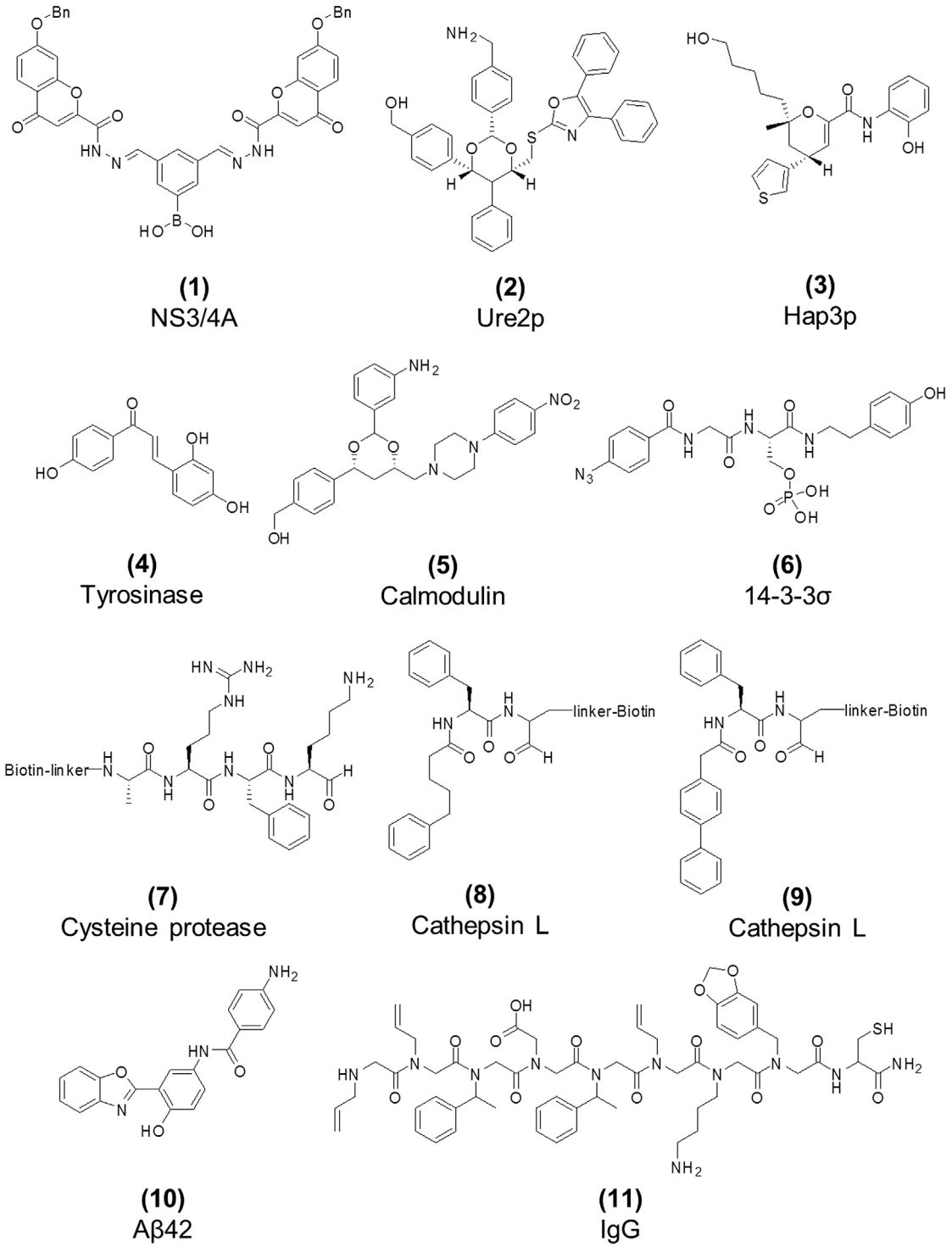

Figure 3. Representative Examples of Small Molecules/Peptides Discovered from Screenings Using Small Molecule and Peptide Microarrays

Each compound is numbered, followed by its corresponding target protein's name.

including small molecules. A key development in SPPS was made in the early 1990s by the introduction of the split-and-pool synthesis concept (Lam et al., 1991; Furka et al., 1991), which made it possible to construct large peptide libraries with a massive number of molecules in a few short steps. With all of these arsenals, chemists now have the necessary chemical tools to make hundreds and even thousands of compounds in a short time, an essential component for the construction of

any kind of microarray. Peptide libraries used in a microarray can be roughly categorized into two subclasses, sometimes without clear distinction: knowledge-based libraries and combinatorial libraries. Knowledge-based libraries refer to peptide libraries constructed using previously known peptide sequences and are routinely employed to investigate proteins having known substrates or interacting partners. Common combinatorial concepts such as positional scanning, alanine scanning, and amino acid deletion can be used to construct knowledge-based peptide libraries. Positional-scanning libraries are constructed by introducing mutations at specific positions within known binding sites of the target protein. This type of peptide library is useful to discover peptide substrates with improved binding activity. In alanine-scanning libraries, each amino acid in the peptide is replaced by an alanine residue to probe the contribution of individual amino acids. To design deletion libraries, the flanking residues are systematically removed from the parent peptide to identify the minimum sequence required for activities. Peptide microarrays made from these libraries have been routinely used to investigate amino acid residues that are critical to molecular interactions (Uttamchandani et al., 2003). Microarrays made of combinatorial peptide libraries, on the other hand, can be more appropriate if the substrate or binding partner of the target protein is unknown. In principle, either semirandom or completely random peptide libraries may be constructed. In practice, the semirandom combinatorial approach is usually carried out in order to minimize the number of peptides needed to be synthesized, and this is done by introducing a series of amino acid building blocks at randomized positions while holding several previously defined amino acid positions fixed. The method can be used to study unknown proteins and identify new peptide substrates/ligands. It also enables a more comprehensive study of protein-peptide interactions. In a recent example, we introduced what we called a fragment-based peptide microarray based on this concept to investigate the substrate specificities of seven 14-3-3 proteins (Lu et al., 2008). 14-3-3 proteins are phosphoserine/phosphothreonine (*p*S/T) binding proteins that interact with numerous cellular proteins and regulate a variety of protein-interacting events. In order to minimize the number of peptide spots and allow sufficient sequence coverage in a heptapeptide, $P_{-3}P_{-2}P_{-1}pS/TP_{+1}P_{+2}P_{+3}$, the two fragments flanking each side of *p*S/T were replaced, one at a time, by degenerated peptide sequences (Figure 2C). By "scanning" fragments (that is, the tripeptides flanking *p*S/T) rather than positions, these combinatorial libraries retained the "neighboring-position effect" associated with protein-peptide interactions. Several highly selective peptides were identified and further validated. Results from these experiments showed that this kind of peptide microarrays is effective in identifying both known and unknown protein-peptide interactions. The same concept was recently extended to the fabrication of a peptide-small molecule hybrid array (Wu et al., 2010).

Small Molecule Libraries

With their diverse biological properties, small molecule libraries provide another rich source for microarray screening. Small molecule arrays are powerful platforms for discovering new protein/small molecule interactions, which will potentially generate lead compounds for drug discovery. By arraying thousands of distinct small molecules on a single slide, the potency and selectivity of small molecule ligands against target proteins can be rapidly evaluated. Several synthetic methods, such as diversity-oriented synthesis (DOS) and fragment-based approaches, have been developed over the years to facilitate synthesis of small molecule libraries. DOS dramatically facilitates the construction of small molecule libraries with both skeletal and stereochemical diversity (Schreiber, 2000). For instance, a 3,780-member small molecule library was constructed by Schreiber's group using a DOS approach (Kuruvilla et al., 2002). The small molecule library shared a common 1,3-dioxane scaffold, which could be synthesized in a stereoselective manner. Upon completion of the synthesis, small molecules could be released from the solid support and anchored covalently onto array. One small molecule identified from array, which was later named uretupamine A (**2**; Figure 3), was found to inhibit Ure2p selectively in a physiological environment. This and other similar DOS-based small molecule microarrays have been used to screen a number of interesting protein targets (haptamide B, **3**, as a Hap3p binder; Figure 3) (Koehler et al., 2003; Barnes-Seeman et al., 2003), some of which will be further elaborated in the following paragraphs.

Protein Libraries

In general, proteins tend to be more fragile and delicate when compared to peptides and small molecules. Consequently, fabricating a protein library has proven to be much more complex and time-consuming. The first proteome array was generated by Snyder and coworkers in the form of a yeast proteome array. The group cloned and expressed 5,800 yeast proteins from 6,200 yeast open reading frames with an oligohistidine tag and subsequently anchored them to nickel-coated slides to perform global proteome analysis (Zhu et al., 2001). With this setup, the group made the first demonstration that novel calmodulin- and phospholipid-interacting proteins can be readily identified from the fabricated proteome array. Later, the same group used this proteome array to carry out large-scale analysis of protein phosphorylation in yeast (Ptacek et al., 2005). Over 4,000 phosphorylation events with 1,325 different proteins have been identified. Novel regulatory modules were discovered by integrating the massive amount of data obtained from different protein-associated events, including protein phosphorylation, protein-protein interaction, and protein-DNA binding. This yeast proteome array, as well as similar proteome array spotted with human proteins, has been commercially available (http://www.invitrogen.com) for several years.

Caveats of Each Type of Library

The construction of peptide libraries is comparatively easier than other types of libraries, and the yield of peptide libraries is much higher than that of small molecule libraries. Because of this, peptide microarrays remain the most popular and effective tools in academic laboratories for large-scale analysis of protein functions and interactions. The information on substrate/ligand specificity derived from peptide microarray data can help to predict physiologically relevant protein-interacting partners and provide useful information for inhibitor design. The interaction between selected peptide sequences and screened proteins, however, may not truly reflect the interactions under physiological conditions. The synthesis of small molecule libraries is not as straightforward as peptide libraries. The synthetic route of small molecule libraries needs to be carefully devised

Table 1. Various Immobilization Methods Developed for Microarray Fabrication

Immobilization Methods	Peptides	Small Molecules	Proteins
Noncovalent	biotin/avidin	fluorous/fluorous	His tag/Ni-NTA
	DNA/DNA	biotin/avidin	ZR/ZE domain
	DNA/PNA		GST/anti-GST
			DNA/DNA
Random covalent	amine/NHS	photocrosslink	amine/NHS
	amine/epoxy	isocyanate/various	amine/epoxy
	amine/aldehyde	silyl chloride/alcohol	amine/aldehyde
Site-specific covalent	Diels-alder	staudinger ligation	native chemical ligation
	native chemical ligation	glyoxylyl/aminoxyl	click chemistry
	Staudinger ligation	tetrazine/dienophile	staudinger ligation
	glyoxylyl/semicarbazide	thiol/quinone methide	oxime ligation
	thiol-ene		

A detailed discussion of different immobilization methods for the three types of microarray can be found in previous reviews (Wu et al., 2011a; Foong et al., 2012). GST, glutathione S-transferase; Ni-NTA, nickel-nitrilotriacetic acid; PNA, peptide nucleic acid.

to introduce multistep diversity and ensure acceptable purity and yield at the end of the synthesis. Stereoselectivity is one of the critical factors to be accounted for during the design of small molecule libraries, as most chiral small molecules can only bind to their intended biological targets with their correct stereoisomers. It poses a substantial challenge for synthetic chemists to design stereoselective reactions that are compatible with solid-phase synthesis. Finally, although protein microarrays have presented themselves as powerful and versatile tools for large-scale proteomic studies, the production of large collections of functional proteins with high purity is still prohibitively expensive and not easily achievable in most academic laboratories at the moment. A number of questions still need to be carefully addressed when carrying out protein microarray research, such as whether the proteins still retain their functions on the array after expression, whether the proteins are functional only when they are in a complex form, and which specific posttranslational modification the proteins carry. Most large-scale productions of proteins needed in a protein microarray thus far do not sufficiently address these critical issues, making data generated from the resulting platform significantly less meaningful than promised.

Microarray Immobilization

Unlike in situ synthesized microarrays, on which biomolecules are immobilized while they are being synthesized on the array, spotted microarrays need a requisite microarray immobilization step upon completion of library synthesis. This is done by depositing biomolecules in small droplets onto suitable surfaces using a robotic dispensing system. Depending on the immobilization strategy used, the molecules can be linked to slides covalently or noncovalently. Immobilization methods are at the heart of most microarray technologies and can significantly affect the quality of downstream microarray screening. Several factors need to be considered when deciding on a suitable immobilization strategy (i.e., molecular orientation, linkage chemistry, and stability of the biomolecules). Numerous immobilization methods have been developed in recent years and have been extensively discussed in our previous reviews (Wu et al., 2011a; Foong et al., 2012). We have summarized these key methods in Table 1 according to the types of biomolecules. The methods can be broadly classified into three types: noncovalent immobilization, random covalent immobilization, and site-specific immobilization. In the following sections, we will briefly discuss each method with a focus on the most recent publications.

Noncovalent Immobilization

A number of noncovalent interactions have been successfully applied to biomolecule immobilization (Table 1). For instance, DNA-DNA interaction was used in an approach developed by Niemeyer and coworkers (Schroeder et al., 2007). In this setup, several biotinylated peptides were first conjugated with streptavidin-DNA complexes. They were then hybridized onto a DNA array through interaction with the complementary DNA strands. Utilizing fluorous interactions, Schreiber's group constructed a small molecule library with a fluorous tag and anchored the small molecules onto array to screen potent inhibitors of the HDAc protein family (Vegas et al., 2007). Recently, Jeon and coworkers have developed a fluorescent tag system by combining fluorous interaction and a coumarin fluorophore tag. The design allows for the evaluation of microarray fabrication in a stepwise manner through fluorescence detection (Jeon et al., 2012). Valles-Miret and Bradley (2011) devised a novel approach by combining a fluorous tag and photochemistry to immobilize any given compound. In this method, fluorous-tagged diazirines were first immobilized onto fluorous slides before small molecules were printed at the same position (Figure 4A). UV irradiation was then applied to generate highly reactive carbene species to covalently link small molecules to the slide.

Random Covalent Immobilization

In covalent immobilization, the linkage formed between biomolecules and the slide surface is more stable and robust than that formed by noncovalent interactions. This will, in principle, lead to higher resistance to harsh wash conditions. Commercially available epoxy-, aldehyde-, and N-hydroxysuccinimide (NHS)-coated slides are often used for immobilization of biomolecules containing amines and other nucleophilic groups present in biomolecules. It should be noted, however, that biomolecules immobilized by these random covalent methods, due to multiple reactive groups on their surfaces, may possess multiple orientations, resulting in nonhomogeneous immobilization and loss of molecular recognition. This is especially true for proteins and peptides. Small molecules can be immobilized by random covalent methods as well. For example, Schreiber and coworkers developed several immobilization chemistries, including silyl chloride/alcohol as well as diazobenzyldiene/phenol and acidic compounds, to covalently anchor small molecules synthesized from DOS methods onto microarrays. Recently, the team has successfully applied isocynanate chemistry to capture small

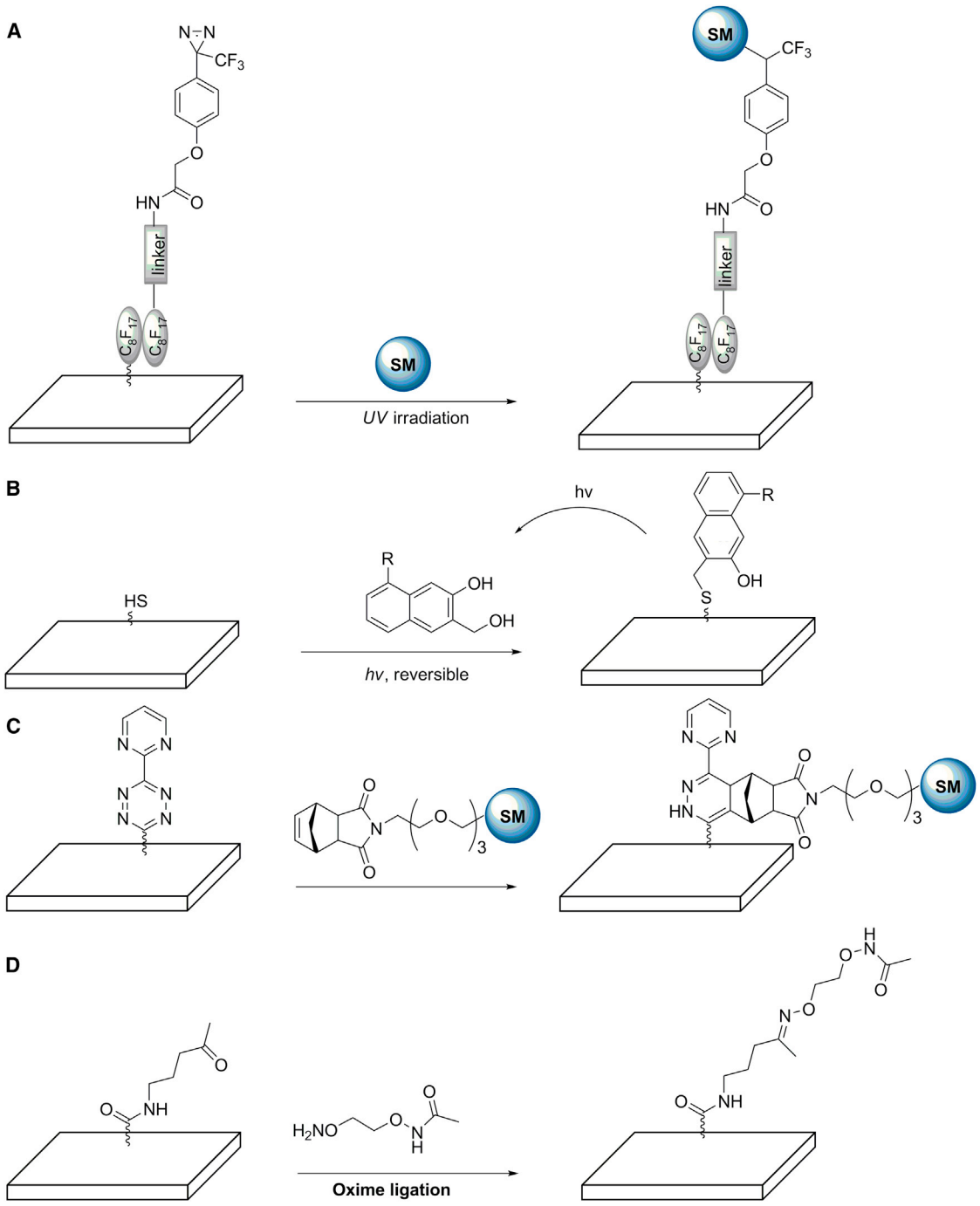

Figure 4. Selected Examples of Immobilization Strategy in Microarray Fabrication
(A) Small molecule immobilization by combining fluorous interaction and photochemistry.
(B) Small molecule immobilization by photochemistry between thiol and quinone methide.
(C) Small molecule immobilization through a reaction between tetrazine and dienophile.
(D) Protein immobilization by combining oxime ligation and EPL strategy.

molecules containing different functional groups, including alcohols, amines, carboxylic acids, thiols, and phenols (Bradner et al., 2006). Park and coworkers immobilized a small molecule library onto an isocyanate-functionalized slide with pyridine vapor activation (Lee and Park, 2011). The group was able to identify 2,4,4′-trihydroxychalcone (**4**; Figure 3) as a novel binder of tyrosinase (K_d = 0.4 μM). The group also found that the slides coated with poly(propyleneoxide) amine (Jeffamine) displayed a higher signal-to-noise ratio compared with slides functionalized by other molecules.

Site-Specific Immobilization

This represents an important advance in microarray fabrication as it allows biomolecules to display in uniform orientations, thereby facilitating molecular recognition. Site-specific immobilization is usually conducted by introducing a unique tag into the biomolecule (Lesaicherre et al., 2002). The biomolecule can then be anchored to slides precoated with appropriate functional groups. A number of classical reactions have been applied by different research groups to immobilize various biomolecules onto arrays, including Diels-Alder reaction, Staudinger ligation, thiol-ene chemistry, native chemical ligation, and others (Table 1). Recently, Arumugam and Popik (2012) developed a reversible light-directed approach for surface functionalization and patterning. This small molecule immobilization strategy is based on photochemistry between thiol and quinone methide (Figure 4B). In this approach, the surface was first photobiotinylated with 3-(hydroxymethyl)-2-naphthol-biotin. It was then stained with a fluorescently labeled avidin. The group found that the thioether linkage was stable under normal conditions. Under UV irradiation, it could be cleaved and regenerate a free thiol molecule. In another recent example, Wittmann and coworkers proved that dienophile-containing carbohydrates could be covalently linked to tetrazine-derivatized slides (Beckmann et al., 2012). To immobilize unfunctionalized carbohydrates, the group devised a bifunctional linker to install a dienophile tag onto carbohydrates through oxime reaction. The carbohydrates with the dienophile tag can subsequently be anchored onto tetrazine-modified slides directly (Figure 4C). The immobilization method was proven to be homogeneous and superior to previous amine- and thiol-based methods. Site-specific immobilization of proteins is never a trivial task because proteins are fragile and may easily lose their activity during the immobilization process. Waldmann and coworkers have recently developed a new strategy based on oxime ligation for site-specific labeling of proteins (Yi et al., 2010). The reaction between oxyamine and ketone is highly specific and efficient. In this approach, a protein was first generated with C-terminal oxyamine through thiolysis of an intein fusion protein. The protein-ONH_2 generated could then react with a ketone-containing fluorophore by oxime ligation. The reaction was shown to be very mild as the protein retained excellent activity after labeling. The group subsequently extended the approach to dual-color labeling and site-specific microarray immobilization of proteins (Yi et al., 2011, 2012) (Figure 4D).

Caveats of Each Type of Immobilization Approaches

Each of these immobilization approaches has its own pros and cons. For example, the DNA-mediated strategy can take advantage of convenient deconvolution techniques using DNA array. However, it requires extra synthetic steps to incorporate a DNA tag into the target molecule. A general consensus for the noncovalent immobilization approach is that noncovalent interactions may not be strong enough to survive subsequent screening procedures. The random covalent immobilization method has provided a convenient approach for microarray immobilization without introducing a specific tag in the molecule. It helps to relieve time and effort during library synthesis. However, it should be noted that biomolecules immobilized using this approach may adopt different orientations on the array, and this may result in loss of protein recognition. In particular, with the photoimmobilization approach, some key interacting groups of small molecules may react with molecular handles on slide surface. The binding site for proteins may therefore be blocked. Site-specific immobilization has undoubtedly provided the most effective approach for biomolecule immobilization. The disadvantage of this approach is that considerable efforts are required to introduce a specific chemical tag to the target molecule during synthesis. This is especially notable in the case of protein immobilization. Nevertheless, the inconvenience of introducing a special tag is paid off by retaining the biological activity of the proteins.

Microarray Application

In the last decade alone, microarrays have evolved from being used primarily as basic analytical research tools into 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. Almost a decade ago, the first microarray-based strategy for rapid and reliable functional annotation of proteins was developed (Chen et al., 2003). The approach uses fluorescently labeled activity-based probes, which detect corresponding enzymes based on their intrinsic enzymatic activity via 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 (Srinivasan et al., 2006; Uttamchandani et al., 2007a). These reports laid the groundwork for potential high-throughput screening of enzymatic activities and inhibition in a protein microarray. Eppinger and coworkers made use of the same strategy to quantitatively determine enzyme kinetics on a microarray (Eppinger et al., 2004). By immobilizing papain (a well-known cysteine protease) on hydrogel slides and incubating it with a fluorescently labeled suicide inhibitor, these researchers were able to obtain kinetic information of the enzyme directly from the resulting microarray data. The strategy was subsequently extended to the study of six cathepsins against seven inhibitors (Funeriu et al., 2005) to obtain the corresponding inhibition constants that were later shown to be consistent with previously reported data. Recently, Jung and coworkers have developed a new surface-concentration-based assay for quantitative kinetic analysis of proteases on microarray (Jung et al., 2012). In this method, a series of peptides with rhodamine

were immobilized onto maleimide-funtionalized slides to derive quantitative kinetic data such as Michaelis constant (K_m) and maximum velocity (V_{max}) using dry-off measurements. By integrating an activity-based probe and an antibody microarray, Cravatt and coworkers took an alternative approach to design a novel microarray platform that enables proteomic profiling of enzyme activities (Sieber et al., 2004); a proteome was first incubated with probes. The labeled enzymes were then captured and anchored onto the antibody microarray to identify the specific enzyme (Figure 5A). Compared with traditional gel-based methods, the array-based method minimized the consumption of expensive reagents and improved the sensitivity to detect enzymes. It should be noted that high-quality antibodies were required for the successful implementation of this approach.

The application of microarray technology has also been extended to the study of functional protein pathways. Lackner and coworkers developed a reverse-phase protein array to analyze the phosphorylation status of 100 proteins with different breast cancer cell lines (Boyd et al., 2008). Cellular lysates from different cell lines were spotted onto the slide in serial dilutions and probed with various antibodies that recognize phosphorylated proteins. The study allowed the group to carry out 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 protein's physiological role and interactions. For enzymes in particular, information about their substrate specificity is extremely critical for a better understanding of their many cellular functions. A comprehensive knowledge of enzyme substrate specificity can also help in the successful design of highly potent and selective inhibitors, ultimately facilitating the drug-discovery process.

Histone peptide microarray has recently become a popular and effective tool in epigenetic research. Epigenetic modifications can have a profound influence on a variety of human diseases. In a recent example, Mrksich and coworkers synthesized a peptide library to investigate the substrate specificities of various lysine deacetylases (Gurard-Levin et al., 2010). The level of deacetylation was analyzed by label-free analysis, in this case MALDI mass spectrometry. The researchers demonstrated that this analytical design was effective in detecting the deacetylation activity of crude cellular lysates and monitoring the changes in the enzymatic activity during the different cell cycles. Arrowsmith and coworkers constructed a position-scanning peptide library on cellulose membrane (a macroarray) based on two histone peptides, H3K9me3 (histone 3 trimethyl lysine 9) and H3K27me3 (histone 3 trimethyl lysine 27), to profile the substrate specificities of chromodomains (Kaustov et al., 2011). In a more recent work by Knapp and coworkers, the researchers synthesized a library of peptides containing all acetylated lysine (Kac) sites from histone proteins on cellulose membranes and investigated the binding preferences of 43 different bromodomains (Filippakopoulos et al., 2012). This study led to the identification of 485 new protein-histone interactions, a number of which were further confirmed by isothermal titration calorimetry. Notably, the study revealed that PTMs could exert significant influence on peptide/bromodomain interactions.

Research on substrate specificity of kinases is another area of active investigation that has been ongoing since the early 2000s. In a more recent study, a microarray containing 290 Tyr peptides and 1,100 Ser/Thr peptides was constructed and used to investigate the substrate specificity of several kinases (Han et al., 2010). With this approach, the group 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 robust tool to facilitate the discovery of potential substrates of other kinases in a high-throughput and sensitive manner.

Our group has made some recent progress in this field by constructing a phosphopeptide array to profile various SH2 domains (Gao et al., 2012). Previous phosphopeptide microarrays had primarily focused on profiling enzymatic activities of different protein phosphatases (Köhn et al., 2007; Sun et al., 2008). 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 were able to successfully pull down target proteins directly from crude cellular lysates. Cellular profiling experiments with different cell lines revealed potential cancer-selective peptides. Further pull-down experiments with these peptide hits led to the identification of three potential cancer biomarkers, highlighting the feasibility of this microarray strategy to facilitate future biomarker discovery.

Computational prediction methods have also been integrated into peptide array for proteome-wide profiling of substrate specificity of proteins. Denu and coworkers utilized SPOT array to screen SIRT3 binders from both known and potential peptide substrates (Smith et al., 2011). 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 and coworkers combined computer modeling and bioinformatics analysis to filter around 700 potential binders of the Abl1 SH3 domain (Xu et al., 2012). These predicted peptides were synthesized, printed onto a microarray, and used to investigate their binding specificities against the Abl1 SH3 domain (Figure 5B). The study indicated for the first time that the Abl1 SH3 domain may interact with numerous methyltransferases and RNA-splicing proteins. This strategy may offer a practical pathway to detect novel protein interactions through domain-peptide recognition events.

Ligand/Inhibitor Binding

Small molecule microarrays are powerful tools to identify potential binders of proteins. Under standard operational procedures, proteins can be labeled with a fluorescent dye (e.g., Cy3 or Cy5) and then incubated with an array of small molecules. Excessive fluorescent dye can be washed away. The detected fluorescent intensity can be used as a guide to identify the small molecule binders of the proteins. A reference protein can be

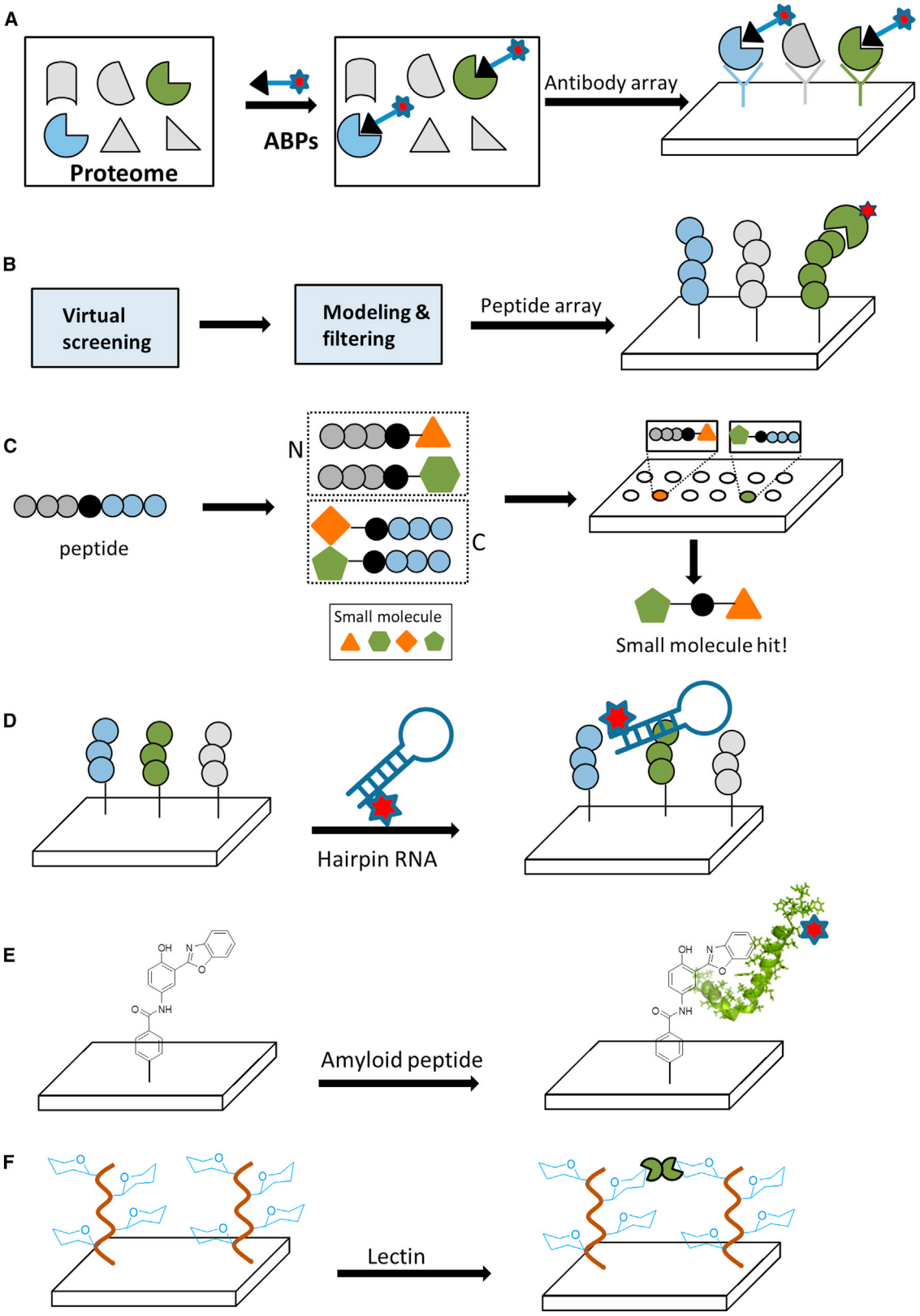

Figure 5. Recent Examples of Microarray Applications in Proteomics
(A) A functional annotation of proteins by integrating the antibody array and activity-based probes.
(B) Substrate fingerprinting of the SH3 domain by integrating the computational method and peptide array.

(legend continued on next page)

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. It should be noted that random dye-labeling methods may interfere with protein function and sometimes can even lead to the denaturation of proteins. To alleviate this problem, several groups have employed a site-directed strategy to introduce fluorophores to proteins through genetically encoded fluorescent tags or affinity tags coupled to dye (Kawahashi et al., 2003; Hurst et al., 2009).

Apart from lead discovery, another important application of small molecule microarray is to generate useful chemical probes, as compounds identified from small molecule microarray screening can be directly converted to chemical probes by simply replacing their immobilization handle with a fluorescent tag without the loss of their activity (Shi et al., 2009). Chemical probes are versatile tools that can help researchers to understand biological functions and the roles of proteins in diseases. Designing and discovering selective chemical probes for a given therapeutic protein has become a highly active research topic in recent years.

Schreiber and coworkers were among the first groups to develop small molecule microarrays for ligand discovery. With their DOS 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 (Wong et al., 2004). One of the compounds identified (**5**; Figure 3) could induce cardiovascular malfunction in a zebrafish phenotypic assay. Recently, the group has anchored more than 15,000 small molecules from a variety of sources onto an array and studied the binding affinity of 100 different proteins (Clemons et al., 2010). It was found that increasing the content of sp3-hybridized and stereogenic atoms in the compound library in general improves the protein-binding selectivity of library members. This finding could yield instrumental information in the future design of compound collections with improved biological activities. By integrating small molecule and peptide hybrid libraries and microarray technology, our group took a different approach to uncover small molecule inhibitors of 14-3-3σ (Wu et al., 2010). In this setup, the two flanking peptide fragments of *p*S residue (RFR*p*SYPP) were replaced by a library of commercially available acid and amine building blocks, respectively. 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. After potent binders from each sublibrary were positively identified, the "hits" amine and acid building blocks were reconstituted to yield the final nonpeptide small molecule inhibitors (Figure 5C). One of the most potent inhibitors identified, **2–5** (**6**; Figure 3), was further tested in cell-based assays and was shown to possess good cell permeability and be capable of inducing apoptosis and cell-cycle arrest of cancer cells. This example underscores the importance of creative compound library design in microarray generation in order to yield novel small molecules with interesting biological activities.

Recently, our group constructed a 270-member peptide aldehyde array to investigate the binding specificities of various cysteine proteases in crude lysates (Wu et al., 2011b). We were able to obtain distinct binding profiles by screening the platform first with fluorescently labeled recombinant proteases. We then moved ahead to test the microarray directly with crude proteome lysates prepared from apoptotic HeLa cells and red blood cells (RBCs) infected with the parasite *Plasmodium falciparum*. Characteristic microarray binding profiles were observed in the study, and they were shown to be directly linked to the endogenous enzymatic activities of cysteine proteases present in both lysates. By screening the RBC-infected cellular lysates further with the platform, we were able to successfully obtain highly distinctive microarray "fingerprints" that differentiate various stages of the parasitic infection. In a further study, the hit-identified ARFK-CHO (**7**; Figure 3) from the array was converted into a chemical probe containing a biotin handle. Several cathepsin proteases were identified in the subsequent pull-down experiment using this chemical probe, again demonstrating the capability of peptide and small molecule microarrays for biomarker discovery. In a more recent work, a small molecule microarray immobilized with 105 aldehyde-containing compounds was screened with mammalian cell lysates overexpressing cathepsin L (Na et al., 2012). Two potent inhibitors, namely D02 (**8**; Figure 3) and D17 (**9**; Figure 3), were discovered from the microarray screening. The inhibitors were later converted to cell-permeable small molecule probes that were used to monitor enzymatic activities in live mammalian cells. The examples summarized herein clearly demonstrate that microarray technology can provide an innovative and rapid approach to the future development of chemical probes.

Other Applications

Apart from the three key groups of proteomic applications mentioned in the previous sections, microarray technology has found other novel applications in several recent examples, which we will summarize in the following paragraphs.

In a very recent expansion of microarray applications, Shin and coworkers developed a peptide array for rapid profiling of peptide-RNA interactions (Pai et al., 2012). A series of peptides were designed based on an amphiphilic peptide, which was previously known to bind strongly to hairpin RNAs. Upon synthesis and immobilization (using an epoxy-modified glass slide), the corresponding peptide microarray was used to screen against six fluorescently labeled hairpin RNAs (Figure 5D). It was revealed that the binding affinity of the peptides was determined by the sequence and the shape of the RNA. A minimum of 14 amino acid residues were required for the peptides to bind to the RNA tightly. Interestingly, one of the peptides identified was capable of inhibiting TAR-Tat interactions in cells. The strategy offers a promising tool to produce peptide-based probes for functional annotation of RNAs in cells.

(C) A ligand binding study of the small molecule array using a fragment-based approach.
(D) The peptide microarray was fabricated to profile peptide/RNA interactions.
(E) The small molecule array was constructed to study the binding specificity of amyloid peptide.
(F) The polymer-glycan array was constructed to profile various lectins.

In another recent example, Wynn and coworkers constructed a high-density overlapping peptide array to map interleukin-13 (IL-13) binding sequence on its receptors (Madala et al., 2011). IL-13 is a cytokine involved in the pathogenesis of allergen-induced asthma. The peptide sequences are derived from two IL-13 receptors, namely IL-13Rα1 and IL-13Rα2. All the peptides consist of 15 amino acid residues, and each of these peptides is selected by shifting three amino acids along the sequence of extracellular domains of receptors. By combining molecular docking and a peptide microarray strategy, the group revealed structural differences between the receptors and successfully generated a receptor-specific antibody of IL-13Rα1.

Hecht and coworkers devised a small molecule microarray to identify amyloid peptide binders (Chen et al., 2010). In this design, a total of 17,905 compounds from various sources, including natural product, commercial compound collections, and DOS library, were immobilized onto slides and screened with fluorescently labeled amyloid peptides (Figure 5E). A total of 79 hits were identified from array experiments and further incubated with PC12 cells to examine their inhibiting activity on amyloid peptide-induced cytotoxicity. One of the identified hits (**10**; Figure 3) was found to enhance fibril formation and unravel a possible novel rescue mechanism without the formation of an early toxic oligomer. The strategy described here may generate useful therapeutic leads to reduce amyloid peptide toxicity and ultimately prevent Alzheimer's disease.

Carbohydrate microarray, a subtype of small molecule microarray, is another field that has been actively pursued. Its application has recently been extended to profile entire organisms. Wong and coworkers have constructed a sialoside microarray to screen different influenza hemagglutinin (HA) subtypes as well as complete viruses (Liang et al., 2011; Liao et al., 2010). It was shown that a minimum of five oligosaccharides are required to discriminate different influenza subtypes, including H1, H3, H5, H7, and H9. The group also discovered that the entire virus shared similar pattern with the HA receptor. In another approach, Bertozzi and coworkers developed a glycopolymer array in which glycans are displayed on a polymer scaffold to mimic native glycans (Godula and Bertozzi, 2012). Different numbers of GalNAc were introduced to the polymer scaffold via oxime ligation. The glycopolymer array generated was screened with four fluorescently labeled lectins. With this approach, the group was able to systematically evaluate the effect of molecular composition and surface density on molecular recognition (Figure 5F). Interestingly, it was found that glycan valency and density can have a dramatic effect on lectin-ligand interactions. The binding preferences will be affected, resulting in different complex formation.

Microarrays can be employed in epitope mapping and serodiagnostic applications as well. Johnston and coworkers devised a random-sequence peptide microarray to explore antibody recognition of sequence space (Halperin et al., 2011). The peptide array consists of 10,000 peptide sequences comprising 17 randomized positions. The peptide sequences were generated randomly in silico, which covers only a small portion of the theoretical sampling space. Individual antibodies were screened with this peptide microarray, and subsequently unique peptide-binding fingerprints were obtained. Subtle antibody-recognition motifs were discovered. It was shown that this platform can be used to predict epitopes of monoclonal antibodies but not of polyclonal antibodies. Waldmann and coworkers recently developed a novel glycopeptide microarray to screen serum antibodies raised against different glycopeptide antigens (Westerlind et al., 2009). A total of 11 mucin peptides with different glycosylation patterns were synthesized in this study. New epitopes were discovered, and the binding patterns of the antibodies raised against different antigens were shown to be unique. This method might provide a valuable tool to investigate antibodies for immunotherapy and immunodiagnostics. To carry out molecular immune diagnostics, Andresen and coworkers constructed a peptide array with 54 peptides from a variety of sources for detecting antibodies in serum (Andresen et al., 2006). The method proved to be highly sensitive and could detect picomoles of antibodies in diluted human serum. Using a peptoid array with 15,000 members, Kodadek and coworkers successfully identified specific IgG biomarkers for Alzheimer disease from serum (Reddy et al., 2011). Notably, the group also uncovered ligands (**11**; Figure 3) that can pull down specific antibodies. The method provides a useful tool to discover the IgG biomarker without knowing the antigens, and it also helps in developing diagnostic assays for various diseases.

Conclusions

We have witnessed numerous innovative and exciting applications of microarray technology in proteomics. With the continuous improvement in library design, surface immobilization, and detection methods, microarray technology has established itself as an effective tool to advance research in biology and medicine. Traditional barriers to acquire diverse library collections have been alleviated by the development of various library-construction strategies, such as DOS for small molecule microarray, fragment-based combinatorial synthesis for peptide microarray, and in situ cell-free synthesis for protein microarray. Label-free detection techniques with improved sensitivity, including mass spectrometry and surface plasmon resonance, have continued to complement the conventional fluorescent labeling methods. Compared with label-based methods, label-free methods do not require protein labeling, thereby retaining the query protein in its native state and minimizing the chance of interfering with protein functions.

One of the greatest obstacles for microarray to gain wider popularity in proteomics is the high cost of the instruments and library resources, which makes the tool inaccessible to many research groups. The quality of the fabricated microarrays and the consistency of microarray data are other important factors that affect the general applicability of this technology. This is especially problematic for protein microarrays. Despite commercialization by several biotech companies, protein microarrays remain a tool used only occasionally in academic labs, primarily due to the cost and more importantly to the unknown functional state and activity of most proteins immobilized on the chip. Comparatively, peptide microarrays are much more reliable and less expensive, and therefore have gained much popularity among proteomic researchers in recent years. Small molecule microarrays, on the other hand, have continued to remain a highly specialized tool, accessible only by select groups of synthetic chemists who have keen interest in proteomics research and drug discovery. Looking forward, we believe that continuous improvement in all facets of microarray technology

will help make microarray data more reliable. This will in turn make the technology better received by a wider scientific community and fuel the further expansion of its applications in proteomics. For example, protein expression array usually suffers from drawbacks like low protein expression and loss of protein activity. Incorporation of common standards and appropriate normalization procedure will help to alleviate the problem. It is also observed that nonspecific binding could often introduce false negatives with the microarray technique. By applying different concentrations of proteins onto the array, the chance of obtaining false-negative binders can be significantly reduced. Alternatively, dual labeling of the query protein under both native and denatured states will also help in identifying the real binders of the proteins (Uttamchandani et al., 2007b).

Despite the aforementioned challenges and barriers, we are optimistic that more innovations in microarray technology will continue in the coming years. The growing number of cloned genes from various species makes it possible to construct different types of proteome microarrays and facilitate molecular interaction network studies across the entire proteome of different organisms. Development of stereoselective synthesis that is compatible with solid-phase chemistry will facilitate the synthesis of a natural-product-like combinatorial library with the desired stereoisomers and generate more therapeutic leads for drug discovery. Further development of novel surface immobilization chemistry will continue to improve the throughput and sensitivity of assays that can be screened on the microarray and cut down the amount of precious biological/clinical samples. With the development of nanolithography and imaging techniques, ''nanoarrays'' with size reduced by several orders of magnitude may also come to life in the near future. Bioinformatics will also help to overcome the restriction of library size in the peptide microarray. Through collaboration with clinical scientists, microarray technology could find more medical applications such as diagnostics and biomarker discovery. Different types of patient sera and tumor extracts can be directly applied onto the microarray to obtain their unique fingerprints for diagnostic purpose. The unique ligands identified from the array can be conveniently used to identify the biomarkers underlying the diseases.

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.

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