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REVIEW

Functional protein microarray: an ideal platform for investigating protein binding property

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Abstract **Functional protein microarray is an important tool for high-throughput and large-scale systems biology studies. Besides the progresses that have been made for protein microarray fabrication, significant advancements have also been achieved for applying protein microarrays on determining a variety of protein biochemical activities. Among these applications, detection of protein binding properties, such as protein-protein interactions (PPIs), protein-DNA interactions (PDIs), protein-RNA interactions, and antigen-antibody interactions, are straightforward and have substantial impacts on many research fields. In this review, we will focus on the recent progresses in protein-protein, protein-DNA, protein-RNA, protein-small molecule, protein-lipid, protein-glycan, and antigen-antibody interactions. We will also discuss the challenges and future directions of protein microarray technologies. We strongly believe that protein microarrays will soon become an indispensible tool for both basic research and clinical applications.**

Keywords lectin microarray, protein microarray, protein-cell interaction, protein-DNA interaction (PDI), protein-protein interaction (PPI)

Introduction

Protein microarrays, also known as protein chips, are miniaturized, parallel and high-throughput analysis systems that are usually formed by spotting down hundreds to thousands of different proteins at high-density on a glass slide (Zhu et al., 2001; Tao et al., 2007; Yang et al., 2011). Inherited the advantages of DNA microarrays, they allow the simultaneous determination of biochemical properties with a variety of analytes only at the cost of a small amount of samples in a single assay.

Unlike DNA oligo microarrays that are usually formed via *in situ* oligo synthesis, protein microarrays are typically fabricated by spotting down proteins onto a substrate slide using a standard contact (MacBeath and Schreiber, 2000; Zhu et al., 2001) or a noncontact microarrayer (Jones et al., 1998; Delehanty and Ligler, 2003; Delehanty, 2004). Contact printing is more popular for fabricating high-content protein microarrays. Quilled metal pins are usually used to deliver subnanoliter of proteins to a slide surface by contact printing, while noncontact dispensing techniques deliver a small droplet of a protein sample to a slide surface without touching it (Roda et al., 2000; Avseenko et al., 2002). For protein immobilization, an ideal microarray surface should be capable of reserving protein conformation, protein activities, retaining more proteins, and possessing very low non-specific background. There are a variety of functionalized substrate slides. On the basis of the immobilizing principle, they can be classified into three types: covalent, affinity, and 3D surfaces (MacBeath and Schreiber, 2000; Kusnezow et al., 2003). On a covalent surface the glass is usually derivatized with aldehyde, epoxy, or NHS moieties that can form a covalent bound with primary amines of a protein. Obviously, proteins are immobilized on the surface in a random orientation. However, when a glass slide is coated with affinity reagents, such as nickel-NTA, GSH-, or streptavidin, proteins with the corresponding affinity tag (*e.g.*, Hisx6, GST, and biotin) are

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more likely to be oriented uniformly on the surface (Jeong et al., 2011). To achieve a maximum amount of proteins absorption on a surface, nitrocellulose- (Stillman and Tonkinson, 2000; Kramer et al., 2004) and PAGE gel-coated glass slides (Angenendt et al., 2002; Charles et al., 2004) have been developed and they do have unique advantages as we will discuss later in this review.

Unlike nuclei acids, proteins have very different biochemical properties, thus, detection of protein binding properties has to be performed under the condition of minimum destructive effect to protein conformation. Fluorescent dye and radioisotopes are the two most frequently used labeling strategies for signal detection (Shingyoji et al., 2005; Zheng et al., 2005; Koshi et al., 2006; Zajac et al., 2007). However, in most of the cases, labeling is a tedious process, because some of the analytes are very difficult or may even unable to be labeled. Thus, the ideal strategy is labeling-free detection. To achieve this, several approaches have been developed recently, including scanning ellipsometry (Carlsson et al., 2005), surface plasma resonance (SPR) (Mecklenburg et al., 2002), resonance light scattering (Gao et al., 2010), and the oblique-incidence reflectivity difference (OI-RD) method (Zhu et al., 2007a).

In 2001, the Snyder group (Zhu et al., 2001) reported a protein microarray composed of 5800 individually purified, unique yeast proteins. Because this microarray covers more than 80% of the proteins encoded by the yeast genome, it formed the first proteome microarray, representing a major breakthrough of the field of protein microarray technology. Recent years have witnessed a flourish of such proteome microarrays in other organisms, such as viruses, bacteria, and humans. In parallel, Snyder, Zhu, and other groups have developed various types of biochemical assays, especially covalent enzymatic reactions, and demonstrated their applications in both basic research and clinical applications (Nielsen et al., 2003; Ptacek et al., 2005). These enzymatic assays developed for protein microarrays have been extensively reviewed elsewhere (Zhu and Snyder, 2001; MacBeath, 2002; Chen and Zhu, 2006; Zhou et al., 2011). Herein, we focus on the latest applications of protein microarrays for the probing of protein binding properties (Kramer et al., 2004; Hamelinck et al., 2005; Popescu et al., 2007a,b; Wingren and Borrebaeck, 2008) (Fig. 1).

Protein-protein interactions

Protein-protein interactions (PPIs) are of central importance for virtually every biologic process. There are numerous technologies for discovering and measuring PPIs. Yeast two-hybrid (Y2H) is a classic technology for screening novel protein-protein interactions. Alternatively, phage display-based screening has also been widely applied for this purpose. The Y2H approach has a tremendous success in

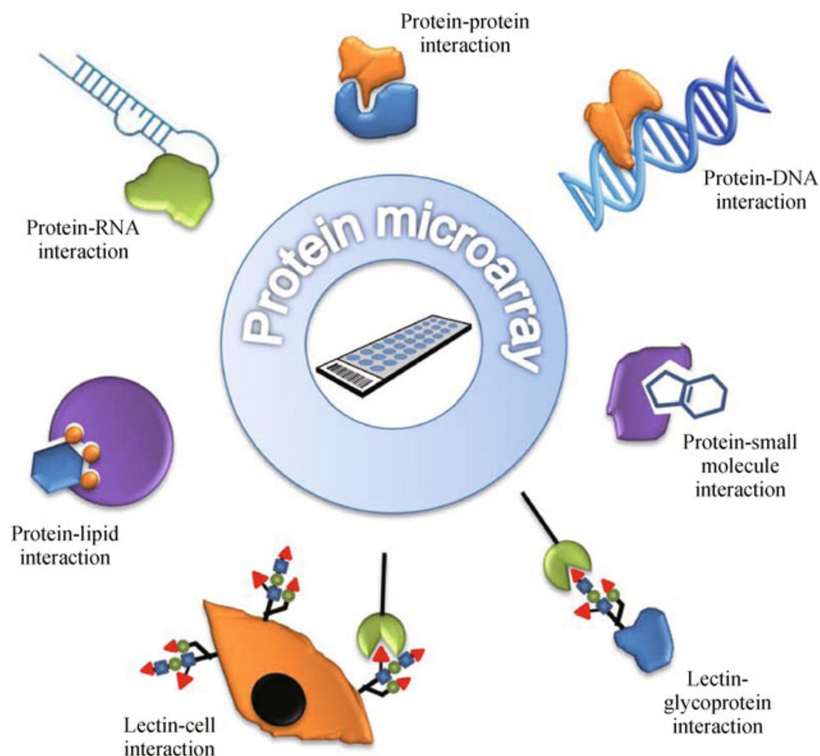

Figure 1 Protein microarrays for protein-ligand interaction study. The ligands could be probed on the protein microarray are protein, DNA/RNA, small molecule, glycan, lipid and etc.

construction of PPI networks in a variety of model organisms; however, they are quite laborious to perform proteome-wide because a minimum of $N^2/2$ combinations of Y2H assays have to be carried out for a given organism encoding a number of N proteins. Furthermore, the associated high false positive rate normally requires reciprocal confirmation assays, which would further increase the labor of Y2H assays substantially. Finally, these approaches only provide binary results — they cannot provide any information about a given PPI affinity value.

On the other hand, the protein microarray technology may serve as a complementary method for PPI screens as it is capable of screening protein-protein and protein-peptide interactions in high-throughput fashion with the capability of quantification.

In general there are three slightly different strategies for studying protein-protein interactions on a protein microarray (Fig. 2). When a highly specific antibody with high affinity against the native protein in question is available, positive PPI events can be detected using a fluorescent conjugated antibody (Fig. 2a). When the protein in question is fused with an affinity tag, such as GST, 6xHis, and V5, antibody against that tag can be used, followed by incubation with a fluorescently labeled second antibody (Fig. 2b). In the last scenario, if a protein in question does not have a specific antibody available and without any tag, the protein can be labeled directly with a fluorescent dye or biotin. However, the labeling efficiency is critical for the final binding results; an optimal labeling assay is normally required (Fig. 2c).

For example, potential binding partners of calmodulin were surveyed against the yeast proteome microarray as reported by Zhu et al. (2001). By incubating biotinylated calmodulin with calcium on the microarrays, six of the 12 known calmodulin binders were successfully identified; of the other 6, two are not in their collection thus not on the protein microarray, and the other 4 are not detectable in the quality control experiment (GST probing). They also discovered 33 novel potential calmodulin binding proteins, and sequence analysis revealed that 14 of the 39 colmodulin binder have a consensus sequence, presumable a novel calmodulin binding motif.

The real power of protein microarrays in PPI studies was demonstrated by MacBeath and colleagues using a protein microarray composed of purified protein domains (Jones et al., 2006). They developed a binding assay that allowed for semiquantitative measurement, and applied it for investigating protein-peptide interactions which play an important role in signaling pathways. Sixty one peptides representing tyrosine phosphorylation sites on four ErbB receptors were incubated with the array containing 159 human Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains. Eight concentrations of each peptide, ranging from 10 nM to 5 mM, were tested in the assay, allowing quantitative measurement of the binding affinity of each peptide to its protein ligand. As a result, 43 previously recognized interactions and 116 new

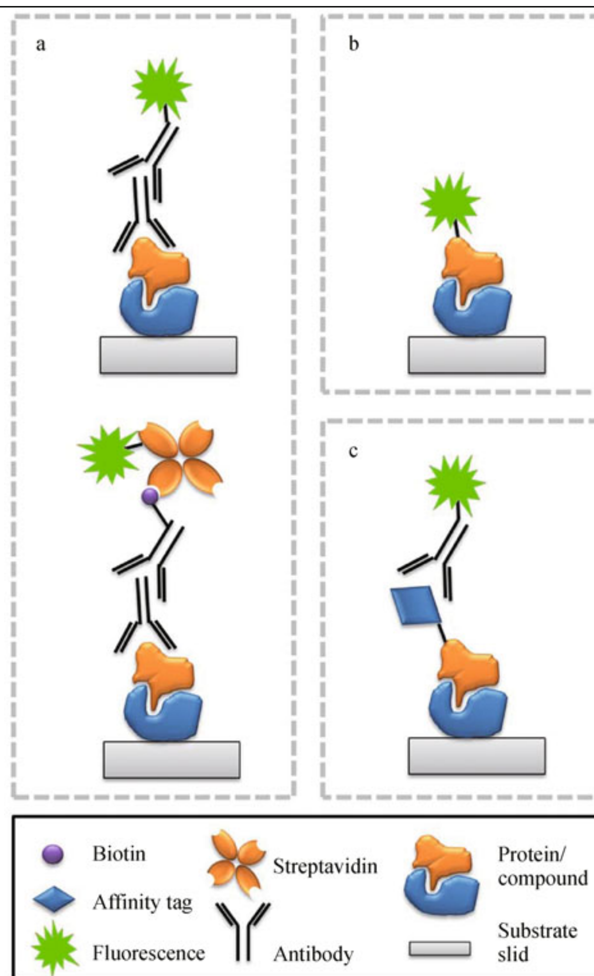

Figure 2 Labeling strategies for protein microarray based PPI study. (a) Bound target proteins are recognized by a specific primary antibody followed by a fluorescent secondary antibody or by a biotinylated secondary antibody. (b) The target protein is prepared as a fusion protein with an affinity tag, thus the binding could be readout by a primary antibody specific for the affinity tag followed by a fluorescent secondary antibody. (c) The target protein could be directly labeled by a fluorescent dye when there is no specific antibody and affinity tag available.

biophysical interactions were found. In addition, they discovered different receptor tyrosine kinases become promiscuous differently when overexpressed. To our knowledge, this systematic study demonstrated that protein microarrays could also be used for quantitative (at least semiquantitative) measurement of protein-ligand interactions.

To evaluate the performance of protein microarrays in PPI study, it is required to compare it side-by-side with other platforms. Tao group selected an apoptosis related protein BAG3 as an example and screened for its binding partners using both a human proteome microarray with >17000 human proteins (Jeong et al., 2012) and a mass spectrometry-based strategy simultaneously. Surprisingly, most of the known binders have been successfully identified by both of the two platforms, and more impressively, more than 50% of the newly discovered BAG3-interacting proteins were

identified as shared targets using both two platforms. These results indicate that protein microarray is comparable with mass spectrometry for PPI study while much less time and labor is required (Manuscript in preparation).

In another application, Popescu et al. developed a protein microarray containing 1133 proteins in *Arabidopsis thaliana* and also applied this microarray for discovering proteins that could bind to calmodulins or calmodulin-like proteins (Popescu et al., 2007a). To identify CaM/CML-interacting proteins, the protein microarrays were probed with Alexa Fluor 647-conjugated CaM1, CaM6, CaM7, CML8, CML9, CML10, CML12 in the presence of calcium, and an Alexa Flour 594-conjugated bovine CaM (BtCaM) was used as a positive control. A total of 173 different proteins that bound the three CaMs and four CMLs were identified. Approximately 25% (44 of 173) of the proteins interacted with all CaMs/CMLs, whereas the same percentage of proteins interacted with only one CaM/CML. The remaining 50% of the proteins bound to two or more CaMs/CMLs. Interestingly, a large number (60 of 173) of CaM/CML-interacting proteins were transcription factors. Bioinformatics analysis showed that CaM binds to several TGA and WRKY transcription factors that play a role in the activation of stress or defense pathways. Furthermore, 20 kinase or kinase-like proteins were also identified, implying that they might be substrates of various CaMs/CMLs.

The latest example of applying protein microarray for systematic PPI study was reported by the Snyder group. To fully and systematically understand the pathways that protein kinases are involved, they purified 85 out of the 122 yeast protein kinases and probe them individually on a yeast proteome microarray (Fasolo et al., 2011). One thousand and twenty three PPIs were successfully identified and most of these PPIs are novel. Further bioinformatics analysis revealed that many of these PPIs could be linked to previously distinct cellular pathways. Overall, their results indicate that kinases operate in a highly interconnected network that coordinates many activities of the proteome.

Protein-DNA and protein-RNA interactions

Protein-DNA interactions (PDIs) play a central role in regulating DNA replication and gene transcription. In general, proteins that directly bind to DNA in a sequence-specific fashion and are capable of regulating nearby gene expression (either activation or repression) are considered as transcriptional factors (TFs) (Teichmann and Babu, 2004; Popescu et al., 2007b). In addition, sequence-specific DNA binding proteins other than TFs may also be very important in DNA replication, DNA repair, and chromosome dynamics (Zhu et al., 2003; Petukhova et al., 2005).

The most intensively studied subset of PDIs is those between DNA binding proteins and their specific DNA target sequences. *In vitro* assays, such as DNA footprinting and gel mobility assays, are frequently applied for this purpose. Recently, chromatin immunoprecipitation (ChIP) coupled with either DNA microarrays (ChIP-chip) or deep sequencing (ChIP-seq) as detection methods have flourished in globally identifying genomic sites recognized by a particular TF *in vivo*. Because of its simplicity, capability of parallel and high-throughput analysis, protein microarrays are also suitable for global profiling of protein-DNA/ RNA interactions. Specific applications include DNA repair protein discovery, DNA motif-protein interaction network construction, and viral RNA-host protein interactions (Fig. 3) (Hall et al., 2004; Chen et al., 2008; Hu et al., 2009).

In the earliest protein-DNA interaction study on a protein microarray, Snyder and colleagues screened for novel DNA binding proteins by probing the yeast proteome microarrays with fluorescently labeled, fragmented yeast genomic DNAs (Hall et al., 2004). Among the ~200 potential DNA binding proteins identified in this study, half of them were previously not known to be able to bind to DNA molecules. Further functional studies (*e.g.*, ChIP-chip, knockout, and EMSA) showed that one of such proteins, Arg5,6 that encodes two catalytic enzymes in ornithine biosynthesis, could also bind to a specific DNA motif and plays a role in the regulation of both transcriptional and posttranscriptional processes. The discovery of such a moonlighting function of Arg5,6 as a TF was only possible with the unbiased global screening, which is one of the key feature of protein microarray technology. Later, the Snyder and Johnston groups have fabricated a protein microarray with 282 known and predicted yeast TFs and probed the microarray with 75 DNA motifs individually (Ho et al., 2006). Over 200 specific PDIs were identified and $>60\%$ of them are previously unknown. Most importantly, the DNA binding activity of Yjl103p, a previously uncharacterized DNA binding protein was confirmed, and many of its target genes were also identified. Further analysis showed that most of these genes are involved in stress response and oxidative phosphorylation.

To identify new DNA damage/repair related proteins, Chen et al. (2008) fabricated a bacterial proteome microarray containing 4256 proteins encoded by the *E. coli* K12 strain, which covering: 99% proteins of the proteome. End-labeled, double-stranded (ds) DNA probes carrying abasic or mismatched base pairs were probed on the microarrays. A small number of proteins were specifically recognized by each type of the probes with high affinity. Two of them (YbaZ, YbcN) were further characterized to have base-flipping activity.

Because of the importance to fully understand transcription regulation, several new approaches have been recently developed to profiling PDI specificity. Bulyk and colleagues developed a ds-DNA microarray that covers the entire DNA 10-mer space and applied it to determine consensus motifs of TFs (Berger and Bulyk, 2009). Wolfe and colleagues applied the yeast/bacteria one-hybrid approach for the same purpose (Meng and Wolfe, 2006). Although highly successful, such

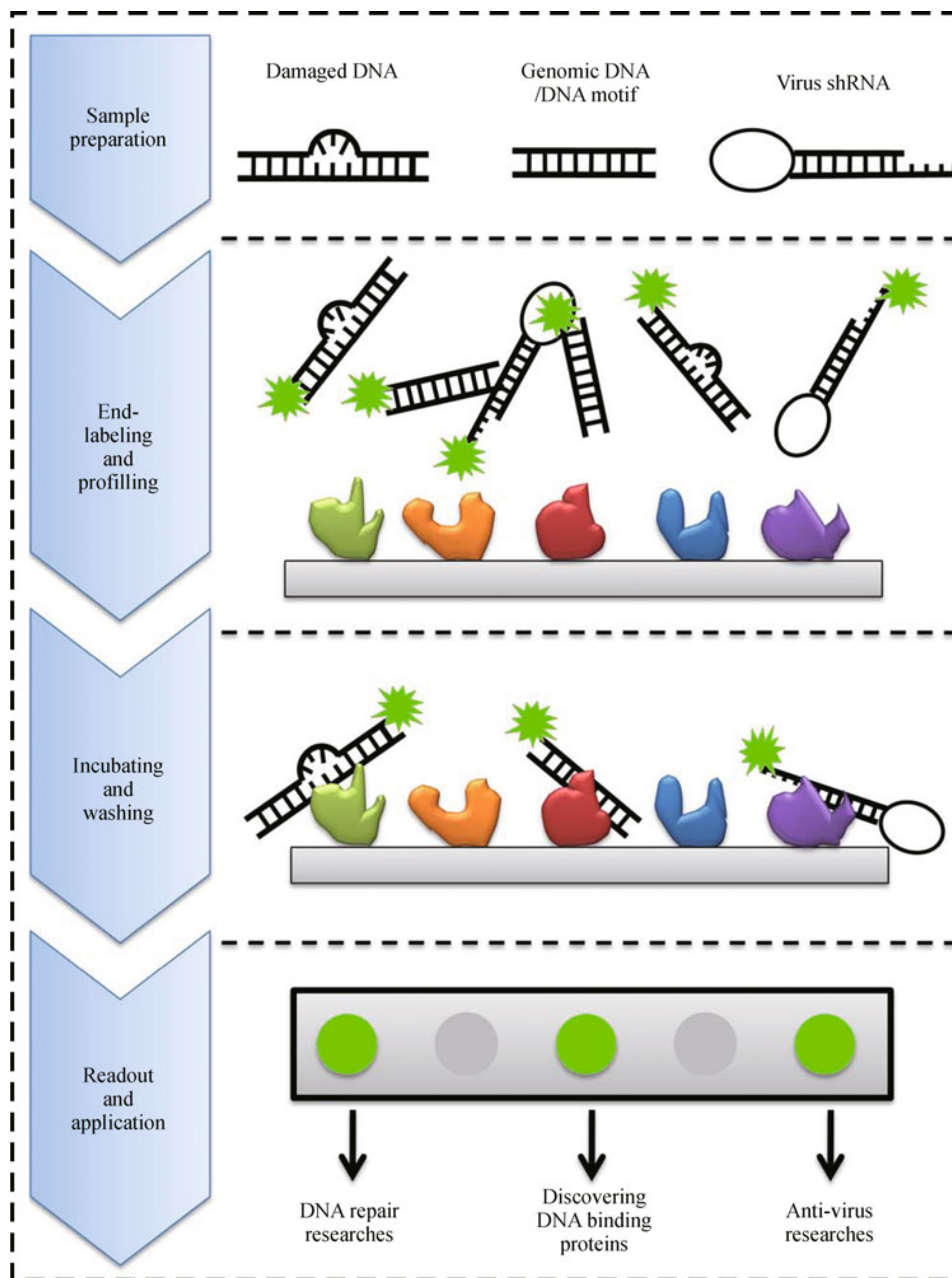

Figure 3 Protein microarrays for protein-DNA/RNA interaction study. Generally, DNA or RNA molecules are end-labeled and profiled on a protein microarray. After washing away the non-specific binding DNA/RNA, the interactions between protein and DNA/RNA can be detected and recorded by a microarray scanner. A variety types of probes such as damaged DNA, DNA motif and virus shRNA could be used as analyte.

DNA-centered approaches require knowledge as which TFs to be studied. Because of this limitation, it becomes quite challenging when the task is to identify potential proteins that specifically recognized a DNA motif of interest, such as motifs predicted to be functional as part of the effort in the ENCODE project (The ENCODE (ENCyclopedia Of DNA Elements) Project, 2004). To solve this problem, Hu et al. (2009) fabricated a protein microarray composed of 4191 full-length human proteins, most of which are TFs, DNA- and RNA binding proteins, chromatin-associated proteins, protein kinases, and mitochondrial proteins. They probed the microarray with 400 predicted and 60 known DNA motifs. 17718 PDIs were thus identified. Many known PDIs for TFs were successfully recovered. A large number of new PDIs for annotated and predicted TFs were also identified. Over 300 proteins that were not previously annotated as TFs were found to show sequence-specific PDIs, including RNA binding proteins, mitochondrial proteins, and protein kinases (Hu et al., 2009; Xie et al., 2010). The most surprising finding is from Erk2, a well studied MAP kinase. Using a series of

well-designed *in vitro* and *in vivo* approaches, they demonstrated that this kinase had a capability of binding DNA independent of its protein kinase activity. Further results revealed it act as a transcription repressor of transcripts induced by interferon gamma signaling (Hu et al., 2009). Again, this study proved the unprecedented unbiased global screening capability of protein microarray for novel functions of the un-characterized proteins as well as the moonlighting function of the well-studied proteins.

In addition to protein-DNA interactions, protein-RNA interactions are also very important for the regulation or inhibition of a variety of biologic functions. To test the feasibility of protein microarrays for protein-RNA interaction study and screen for RNA binding proteins with antiviral activities, a fluorescently tagged small RNA hairpin containing a clamped adenine motif, which is required for the replication of brome mosaic virus (BMV) was incubated with yeast protein microarray (Zhu et al., 2007b). The rationale for using yeast proteome microarray is that this virus can also replicate in *Saccharomyces cerevisiae*. Two of the candidates, pseudouridine synthase 4 (Pus4) and the actin patch protein 1 (App1), were further validated in *Nicotiana benthamiana*, the results showed that they can dramatically inhibit the spread of BMV in plants.

Protein-small molecule interactions

Here we refer small molecules as drug or drug candidates. Usually, it costs more than one billion US dollars and take 12–15 years to bring a new drug to the market. Lots of potential compounds even failed at the final step, *i.e.*, phase III clinical trial. Ideally, if we can correctly filter out the hopeless candidate compounds as many as possible at the early stage, much of the cost and time could be saved, thus making the drug development process more fast and effective. One possible solution for this is to profile the binding proteins of a given compound and try to understand its molecular mechanism on a systems level. This may also be useful for "old-drugs" as to explore the possibility for new treatment/s and to avoid its side-effects.

Among many available technologies, protein microarrays may be the best choice for identifying the compound-targeting proteins globally because of its simplicity (Fig. 4). Huang et al. (2004) incubated biotinylated small-molecule inhibitors of rapamycin (SMIRs) on the yeast proteome microarrays, and identified several target proteins of the SMIRs, including Tep1p and Ybr077cp (Nir1p). Both of them were validated *in vivo* and found to be associate with PI(3,4) P2, which is a second messenger involved in membrane translocation of a variety of cell growth and survival signals. This suggests a novel mechanism by which phosphatidylinositides might modulate the target of rapamycin pathway.

The labeling of the compound is critical for the success of the protein microarray based small molecule-protein interaction studies. Before probing on the microarray, the compound has to be chemically conjugated with a biotin, a fluorescent dye or a radioisotope. However, the radioisotope labeled form is not available for most of the compounds. Even biotinylation and fluorescent conjugation are difficult for the majority of the compounds, because there is either no appropriate site for labeling or the stability and original activity is ruined after labeling, or the labeling cost is unacceptable. The ideal solution to solve this difficulty is to combine protein microarray with a label-free technology. Bore this in mind, Kenyon *et al*. coupled ligand-protein binding assays on a protein microarray and the MS/MS technology as the detection method to detect antibody-epitope interactions. Although this approach is still in its infancy, we expect that it will have a tremendous impact on drug target discovery when MS/MS sensitivity is further improved (Fig. 4 right) (Evans-Nguyen et al., 2008).

Lectin-glycan interactions

As one of the most diverse posttranslational modification (PTMs) of proteins, glycosylation is estimated to modify over 50% of proteins expressed in eukaryotes (Apweiler et al., 1999). In particular, most of the plasma membrane-localized and secreted proteins, which encompass about one third of eukaryotic proteomes, are heavily glycosylated. Thus, cell surface is coated with thousands of glycans, which mediate a variety of biologic processes, such as cell-to-cell communication, cell-matrix interactions, host-pathogen interactions, development, tumor invasion and metastasis. Alters in the composition or structure of these glycans may reflect the dramatic changes in cell phenotype and signaling transductions inside cells. These changes could even cause diseases, such as the I-Cell disease (Kollmann et al., 2010) and leukocyte-adhesion deficiency type II (LAD II) (Gazit et al., 2010).

There are a handful of tools and technologies that can be applied for glycosylation study, *e.g.*, liquid chromatography (LC) (Hase et al., 1978; Tomiya et al., 1988), mass spectrometry (MS) (Kameyama et al., 2005), capillary electrophoresis (CE) (Kamoda et al., 2006; Kamoda and Kakehi, 2006), and flow cytometry (FCM) (Frojmovic et al., 1991; Poulain et al., 1999). However, none of these traditional approaches could meet the criteria of an ideal glycosylation study platform, which is economical, miniaturized, sensitive, and high-throughput. Borrowing the idea of protein microarrays, several groups have developed a powerful tool for profiling cell surface glycans by spotting down lectins, a group of proteins that specially recognize various types of glycans, in microarray formats (Angeloni et al., 2005; Pilobello et al., 2005; Zheng et al., 2005) (Fig. 5).

Kuno et al. (2009) developed an antibody-assisted lectin

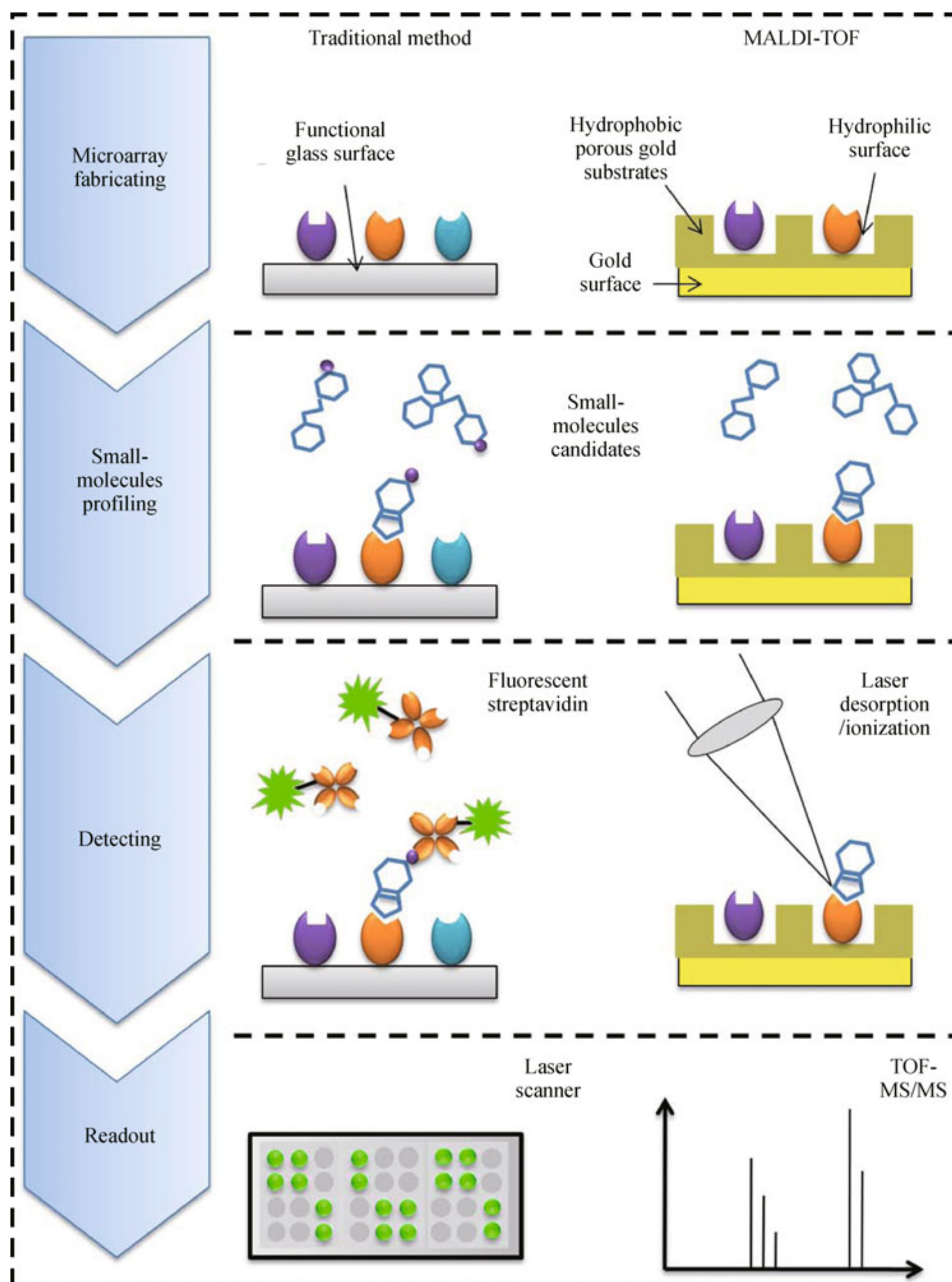

Figure 4 Protein microarrays for protein-small molecule interaction study. Usually, a small molecule such as drug candidate is biotinylated or fluorescent conjugated and probed on a protein microarray. As to overcome the shortcoming of chemically labeling of small molecule prior to probing, MALDI-TOF may be applied as a readout technology. Briefly, a protein microarray is fabricated on a hydrophilic/hydrophobic patterned substrate slide. A mixture of un-labeled small molecules could be probed on this microarray, the results were then readout by MALDI-TOF analysis.

profiling (ALP) to detect glycoproteins at very low concentration in clinic samples using lectin microarrays. Using ALP, proteins were enriched by aid of the appropriate antibodies, which made it possible to be analyzed by a lectin array. They applied this method to analyze the glycan structures of protein hPod, which has been proposed to enhance the metastatic potential of glioblastoma cells. Trying to establish a wash-free detection platform on lectin microarray, Hirabayashi's group also developed evanescent-field activated fluorescence detection system (Kuno et al., 2005; Uchiyama et al., 2006), on which the interactions between the analyte and lectins are monitored on the glass surface immersed under buffer that enables real-time detection of fluorescently labeled glycans. Since the evanescent field is generated within 200 nm, the background level is extremely low and washing steps can be omitted.

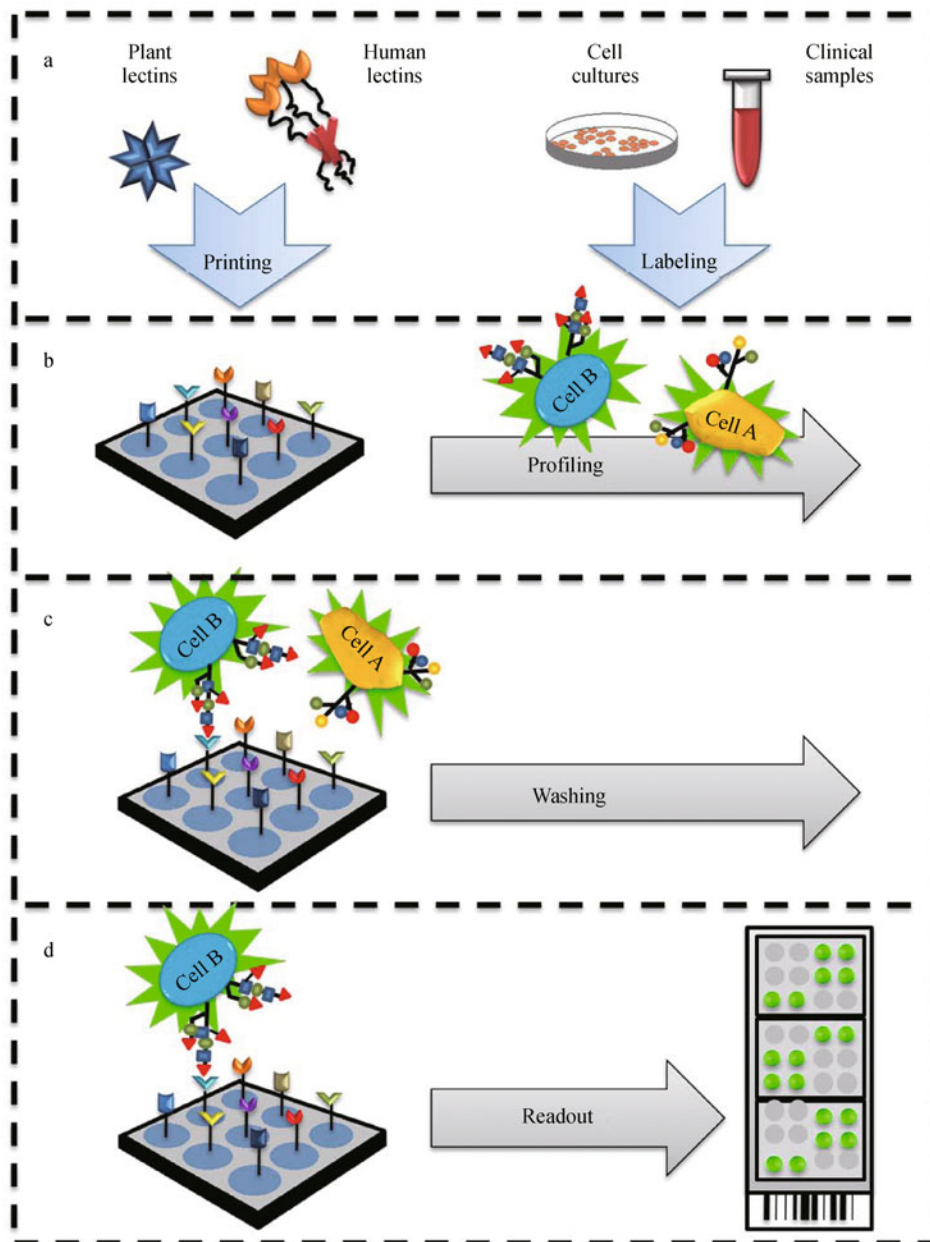

Figure 5 Live cell surface glycan profiling using lectin microarray. Live cells from cell cultures or clinical samples are labeled with a fluorescent dye, such as CFSE. The labeled cells were probed directly on a lectin microarray. After incubation for a while, a mild washing step should be taken to remove the non-specific bindings. The positive binding could be readout and recorded by a high-resolution fluorescence scanner.

To analyze the interactions between protein and glycoproteins in a higher level, intact cells including bacteria, fungi (Kuno et al., 2005; Hsu and Mahal, 2006; Hsu et al., 2006), and mammalian cells were profiled on lectin microarrays, by which the dynamics of cell surface glycosylation during growth, and after stimulation were monitored (Pilobello et al., 2005; Ebe et al., 2006; Chen et al., 2007; Pilobello and Mahal, 2007; Tateno et al., 2007) (Fig. 5).

In the first case of detecting living cells using lectin microarray, Hsu et al. fabricated a microarray with 21 lectins to analyze closely related *E. coli* strains, the nonpathogenic strains JM101 and HB101 and the pathogenic strains RS218. The results revealed that pathogenic bacterial strains had distinct binding signals and different binding patterns from those of non-pathogenic trains, which could provide a surface glycan fingerprint of bacteria (Hsu and Mahal, 2006; Hsu et al., 2006).

However, more exciting results came from the studies of the mammalian cell surface glycans. In 2007, Ebe and Tateno found significant differences in carbohydrate expression on

normal Chinese hamster ovary (CHO) cells and lymphatic endothelial cells (LEC) with their different glycosylation defective mutants using a microarray with 43 lectins (Ebe et al., 2006; Tateno et al., 2007). In another interesting study, normal and tumorigenic human breast cell lines including their sublines differing in the tendency to "home" to different tissues during metastasis were analyzed by lectin microarray and significant differences in carbohydrate expression were observed (Chen et al., 2007).

In 2008, a lectin microarray with 94 different lectins was developed by Tao et al. To qualitatively profile the lectin binding specificity of 24 human cell lines, they developed a binary algorithm that generates a "glycocode" for each cell lines. This has allowed them to hierarchically cluster these cell lines on the basis of their accessible glycan composition. Importantly, they also successfully identified three new lectin biomarkers (LEL, AAL and WGA) that differentiate breast cancer stem-like cells from their parental cell line MCF7. To further verify the results, an murine model was tested, the results showed that lectin-enriched cancer stem cells from MCF7 cell population could indeed produce bigger and more aggressive tumors (Tao et al., 2008). This study is the first case to show the potential of lectin microarray for live cell specific marker identification.

In a most recent study, Tateno et al. (2011) fabricated a lectin microarray with 96 lectins and performed a comprehensive analysis of 114 types of human induced pluripotent stem cells (iPSCs), which were generated from 5 types of somatic cells. Nine human embryonic stem cells (ESCs) were also included in this lectin microarray probing. Data analysis showed that using the glycan binding profiles, the undifferentiated iPSCs and ESCs could be clustered as one large group, which was clearly different from that of the differentiated SCs. Further analysis showed that the expression profiles of relevant glycosyltransferase genes agreed well with the lectin microarray results. These results indicated that lectin microarrays could also be used as a reliable tool to differentiate highly related cells during differentiation based on their glycan profiles.

Protein-lipid interactions

Protein-lipid interaction is an essential feature of biologic membranes. The protein-lipid interactions could also be assayed on a protein microarray. However, due to the hydrophobicity of lipids, they could not be probed directly. They should be assembled into a liposome, which acts as a carrier. Usually, each liposome contains phosphatidylcholine (PC), target lipids and biotin or fluorescent DHPE for results readout. Alternatively, the fluorescent dye can also be packed inside the liposomes (Fig. 6). In 2001, Zhu et al. (2001)used five types of PI liposomes which containing 5% (w/w) PI(3)P, PI(4)P, PI(3,4)P2, PI(4,5)P2, or PI(3,4,5)P3 respectively to probe the yeast proteome microarray. As a result, they identified more than 150 proteins binders of these phospholipids, more than 50% of which were previously unknown as membrane-associated proteins. In a recent follow up study, the Lemmon group further analyzed those unconventional phospholipid binding proteins identified in the above study, and found a new KA domain among three kinases, Kcc4, Hsl1, and Gin4. Mutation in the KA domain impairs membrane association of the intact proteins and reveals the importance of phosphatidylserine for bud neck localization of yeast Kcc4p (Moravcevic et al., 2010). These results established protein microarray as a reliable tool for global protein-lipid interaction study, as to improve the applicability of this tool, protocol for making liposomes with higher stability and more intensive labeling (fluorescence dye or biotin) is highly anticipated. More recently, Chen and colleagues developed an non-quenchable liposome as a carrier to identify yeast proteins that can specifically bind to PI(3,5)P2 using the yeast proteome microarrays (manuscript submitted).

Antigen-antibody interactions in biomarker discovery

Because the majority of proteins present in a purified form on a proteome microarray, they are ideally suited for examining antibody specificity, which is the biggest challenge for traditional antibody generating. In principle, a highly specific antibody should recognize only its target among the other thousands of proteins on the microarray, and when it does not, its cross-reactivity to non-specific targets can be revealed. This idea was first tested by Michaud et al. who probed the yeast proteome microarrays with 14 different commercial antibodies: 6 monocolonal antibodies (mAbs), 6 anti-peptide polyclonal antibodies, and two polyclonal antibodies raised against full length proteins (Michaud et al., 2003). The results were quite astonishing: the mAbs exhibited higher specificity than their polyclonal counterparts, while the anti-peptide polyclonal antibodies were more specific than those against full-length proteins. However, even some mAbs showed some off-target reactivity. This study raised the importance of using proteome microarrays to identify mono-specific antibodies. The use of protein microarrays to profile antibody binding profiles has prompted two other interesting applications in mAb generation (Zhu et al., 2007a; Jeong et al., 2012). In the past, complex biologic samples, such as cancer tissues, have been used as antigens to immunize mice to develop antibodies specific for the diseases. However, this approach only had limited success because it demands additional steps to identify the corresponding antigens, either by screening a cDNA expression library or immunoprecipitation (IP) coupled with MS. In a report by Hu et al. (2007) a human protein microarray composed of 1000 individually

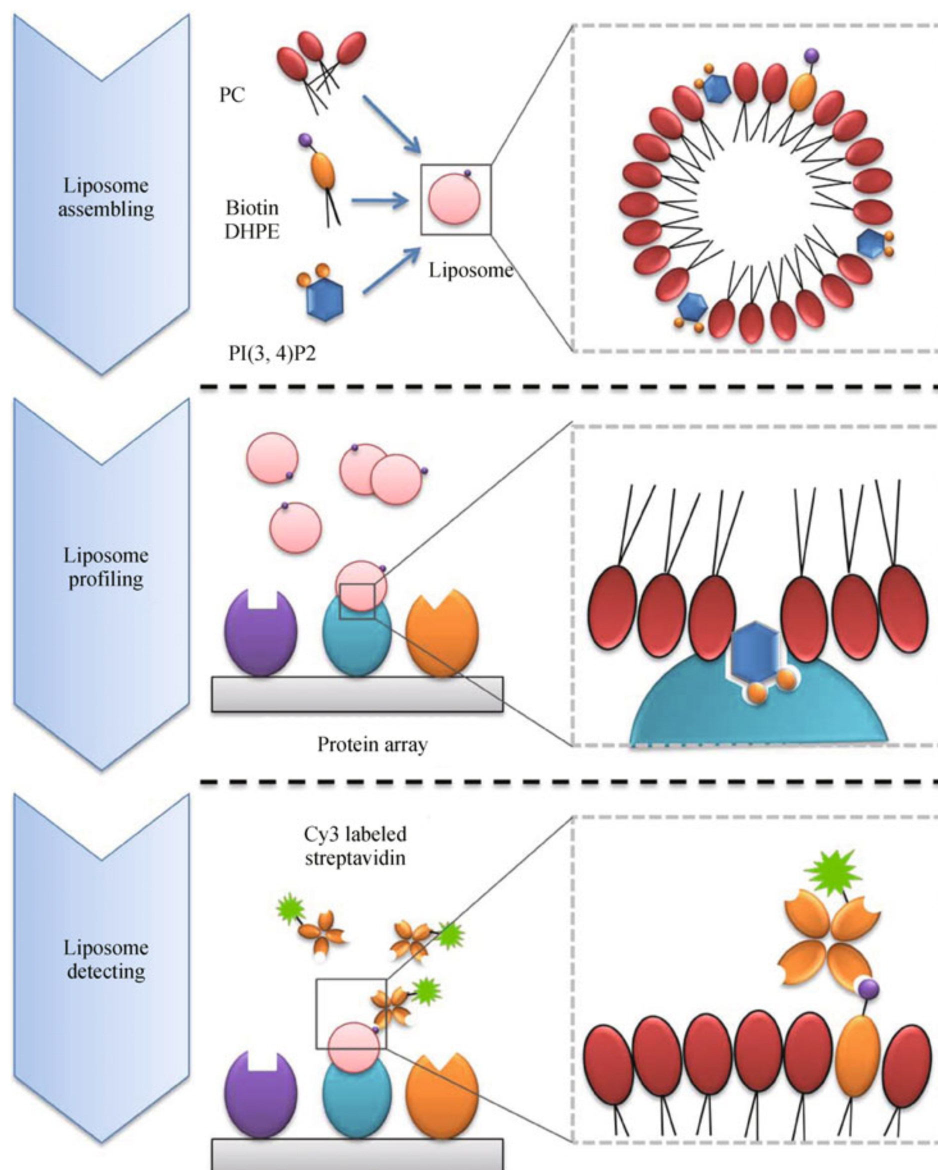

Figure 6 Protein microarrays for protein-lipid interaction study. A target lipid is assembled into liposome with a biotinylated DHPC or a fluorescent dye. Except the sample preparation, the protein microarray based procedure for protein-lipid interaction study is similar to that of protein-small molecule study.

purified proteins were used to determine antigens of hybridomas generated via immunizing mice with live cancer tissues. The protein microarrays serve not only as a tool to deconvolute antigen-antibody interactions, but also as a way to estimate specificity of the obtained mAbs, though among a limited number of 1000 proteins. This idea was further streamlined in a report by Jeong et al., where the human proteome microarrays composed of ~17000 full-length proteins were used to deconvolute mAbs generated with live cell immunization (Jeong et al., 2012). By performing a rather thorough characterization of these mAbs, they found that a large fraction of mMAbs are both of immunoblot- and immunoprecipitation-grade.

Perspectives

Protein microarray technology has been shown to be very useful for multiplexed detection and proteomics studies, especially for protein-ligand interaction studies. Novel applications utilizing protein microarrays and new protein microarray technologies are continually emerging. However, there are still several disadvantages that need to be overcame before protein microarray technology could be widely applied for protein-ligand interaction studies.

First, the availability of protein microarray is still an issue. The traditional cloning-expressing-purification-printing approach is still the gold standard for making protein

microarrays, especially for proteome microarrays. Because of the sophisticated expertise required and the high cost of production, it is almost impossible for most laboratories to make their own microarrays, but the prices for the commercial protein microarrays are unacceptably high. A variety of promising strategies have already been tested to bypass the traditional procedure (Ramachandran et al., 2004; Tao and Zhu, 2006; He et al., 2008). However, none of them are close to be applied for large-scale fabrication of protein microarrays. Thus, to make protein microarray technology more applicable, a simpler and more powerful strategy is needed. Before an alternative strategy can replace the current one, we envision that the current strategy will still be the mainstream in the near future. The foundation of the traditional strategy is the high-quality and expression-ready ORF collections. However, there are only a handful of such collections, such as *E. coli* (Ogura et al., 2006; Chen et al., 2008), yeast (Zhu et al., 2001; Gelperin et al., 2005; Fasolo et al., 2011), human (Invitrogen, ProtoArray), *Arabidopsis thaliana* (Popescu et al., 2007a, 2009). To accelerate biologic research and clinical application, such as host-pathogen interactions (Zhu et al., 2007b; Li et al., 2011; Shamay et al., 2012), expression-ready ORF collections for other important species, such as mouse, rice, infectious pathogens like *Mycobacterium tuberculosis* and etc, are highly desired.

Secondly, there are no commonly accepted standard experimental protocols or data analysis procedures for protein microarray experiments. High variations are frequently observed for a variety of applications, this phenomenon is very similar to what DNA microarrays have encountered at its early stage. To solve this critical issue, we can learn from DNA microarray to set controls for all the key steps and try to get the procedure as standardized as possible. This issue is now being investigated by the Human Proteome Organization (HUPO), and they are developing guidelines for experimental design and data annotation.

Third, at present most of the protein microarray results are, at best, semiquantitative. To reach the goal of accurate quantification, three steps can be taken, *i.e.*, standardizing the whole fabrication and application process, adding quantitative controls to the key steps, and developing new technologies and data processing algorithms.

Though protein microarray technology is still in their infancy, it will no doubt to be one of the most powerful tools in both basic research and clinical application, especially for protein-ligand interaction studies. We expect that the use of protein microarrays to characterize protein binding properties to other biomolecules, such as miRNA, ncRNA, and protein complexes, will flourish in the near future.

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Abbreviations

PPI: protein-protein interaction; PDI: protein-DNA interaction; GSH: Glutathione; NHS: N-Hydroxysuccinimide; SH2: Src homology 2; PTB: phosphotyrosine binding; TF: transcriptional factor ; ChIP: chromatin immunoprecipitation; BMV: Brome Mosaic Virus; SMIR: small-molecule inhibitors of rapamycin; MALDI-TOF: matrix assisted laser desorption ionization/time of flight; PTM: posttranslational modification; ALP: antibody-assisted lectin profiling; iPSC: induced pluripotent stem cell; ESC: embryonic stem cell; PC: phosphatidylcholine; DHPE: N-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; HUPO: Human proteome organization;

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