Protein Microarrays

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Introduction

Protein microarrays, an emerging class of proteomic technologies, are fast becoming critical tools in biochemistry and molecular biology. Two classes of protein microarrays are currently available: analytical and functional protein microarrays. Analytical protein microarrays, mostly antibody microarrays, have become one of the most powerful multiplexed detection technologies. Functional protein microarrays are being increasingly applied to many areas of biological discovery, including studies of protein interaction, biochemical activity, and immune responses. Great progress has been achieved in both classes of protein microarrays in terms of sensitivity, specificity, and expanded application.

Protein microarrays, also known as protein chips, are miniaturized and parallel assay systems that contain small amounts of purified proteins in a high-density format (1). They allow simultaneous determination of a great variety of analytes from small amounts of samples within a single experiment. Protein microarrays are typically prepared by immobilizing proteins onto a microscope slide using a standard contact spotter (1,2) or noncontact microarrayer (3–5). A variety of slide surfaces can be used. Popular types include aldehyde- and epoxy-derivatized glass surfaces for random attachment through amines (2,6), nitrocellulose (7,8), or gel-coated slides (9,10) and nickel-coated slides for affinity attachment of His6-tagged proteins. The last type was reported to provide 10-fold better signals than those obtained with other random attachment methods (1). After proteins are immobilized on the slides, they can be probed for a variety of functions/activities. Finally, the resulting signals are usually measured by detecting fluorescent or radioisotope labels. The typical image of protein microarrays is shown as Figure 1.

Analytical protein arrays can be used to monitor protein expression levels or for biomarker identification, clinical diagnosis, or environmental/food safety analysis. Functional protein microarrays have many uses: (i) to probe for various types of protein activities, including protein-protein, protein-lipid, protein-DNA, protein-drug, and protein-peptide interactions; (ii) to identify enzyme substrates; and (iii) to profile immune responses, among many others. Applications of both the analytical and functional protein microarrays are depicted in Figure 2. In the

following sections, we will provide examples of various applications of both types of microarray, with an emphasis on functional protein microarrays. Given the large volume of papers related to protein microarray technology, we regret that we are unable to cite all the published work in the field.

Analytical Microarrays

Perhaps the most representative class of analytical microarrays is the antibody microarray, in which antibodies are arrayed on glass surfaces at high density. The biggest challenge associated with antibody microarrays is that of producing antibodies that are able to identify the proteins of interest with high specificity and affinity in a high-throughput fashion. Because the traditional method for generating monoclonal antibodies is time-consuming and laborious, researchers have recently sought alternative approaches. For example, phage antibody-display, ribosome display, systematic evolution of ligands by exponential enrichment (SELEX), messenger RNA (mRNA) display, and affibody display have been developed to expedite the production of antibodies with high specificity (11–14). All of these methods involve the construction of large repertoires of viable regions with potential binding activity, which can be selected by multiple rounds of affinity purification. The binding affinity of the resulting candidate clones can be further improved using maturation strategies. However, the ideal selection system is yet to be fully developed: one that is not only fast, robust, sensitive, and of low cost, but also automated and minimized (13,14).

Figure 1. A typical protein microarray image. A yeast protein microarray is probed with anti-GST antibodies followed by detection with Cy5-conjugated secondary antibodies. An enlarged image of one of the 48 blocks is depicted below the protein chip.

Figure 2. Applications of protein microarrays. Antibody arrays can be used for clinical diagnosis or environmental/food safety analysis. Functional protein arrays are mainly used to study various types of protein activities, including protein-protein, protein-lipid, protein-DNA, protein-drug, and protein-peptide interactions, to identify enzyme substrates and to profile immune responses.

Despite the challenge involved in obtaining specific antibodies, many studies using antibody microarrays have recently been reported. In a pioneer work by Haab and colleagues (15), the first high-density antibody microarrays were used to test whether a linear relationship could be detected between an antibody and antigen pair in an array format. They investigated the ability of 115 well-characterized antibody-antigen pairs to react in high-density microarrays on modified glass slides: 30% of the pairs showed the expected linear relationships, indicating that a fraction of the antibodies were suitable for quantitative analysis. Sreekumar and coworkers (16) created antibody arrays with 146 distinct antibodies against proteins involved in the stress response, cell cycle progression, and apoptosis and used these arrays to monitor the alterations in protein quantity in LoVo colon carcinoma cells. The reference standards and samples were labeled separately using either CyTM5 or Cy3 dyes, and the fluorescent signals of the bound proteins were detected with a confocal microarray scanner. These investigators were able to obtain differential expression profiles, with radiation-induced up-regulation of apoptotic regulators, such as p53, DNA fragmentation factors, and tumor necrosis factor-related ligand.

In order to increase affinity and specificity, analytical microarrays usually employ a signal amplification system and sandwich assay format, in which the first antibody is spotted on the array and then a captured antigen on the chip is detected with a second antibody that recognizes a different part of the antigen (Figure 3). A highly sensitive antibody microarray system combining both methods has been shown to be capable of simultaneously detecting 75 cytokines with high specificity, femtomolar sensitivity, a 3-log quantitative range, and economy of sample consumption (17). Although the sandwich format dramatically increases the specificity of the antigen detection, it requires at least two high-quality antibodies for each antigen that is to be detected.

Functional Protein Microarrays

Functional protein microarrays have recently been applied to many aspects of discovery-based biology, including protein-protein, protein-lipid, protein-DNA, protein-drug, and protein-peptide interactions. Although we have attempted to describe all the major applications of functional protein microarrays, it is impossible to cover all the instances in which they have been used. Therefore, we have chosen to focus most of our examples on yeast proteome microarrays (Figure 4).

Protein-Protein and Protein-Lipid Interactions

Zhu and coworkers (1) reported the construction and application of the first proteome microarrays, which contained >5800 individually purified yeast proteins or 85% of the yeast proteome. These proteome chips were first used to study protein-protein interactions, in which the chips were incubated with biotinylated calmodulin in order to identify its new binding partners. In addition, protein microarrays were also used to examine interactions with various phospholipids, which are known to act as secondary messengers. When biotinylated liposomes containing various phospholipids of interest were used as probes, more than 150 phospholipid binding proteins were identified, and a wide range of proteins was found to bind to the lipid vesicles; over 50% of these were previously known to be associated with membranes.

Protein-DNA Interactions

In a later report, the same research group also used the chips to screen for novel DNA binding activities using fluorescently labeled yeast genomic DNA (18). A total of 200 proteins that reproducibly bound DNA were identified. Half of them had not previously been shown to have DNA binding activity; these new proteins fell into a wide variety of functional categories. The most surprising discovery in this category was the identification of Arg5,6 as a DNA binding protein. The *ARG5,6* gene encodes two mitochondrial enzymes that mediate two key steps in the biosynthesis of ornithine (a precursor to arginine). Follow-up experiments revealed that this enzyme associates with specific mitochondrial loci in vivo, and this information was used to define a DNA binding motif for this protein. Thus, a novel DNA binding activity was found to be associated with a well-characterized protein, thereby identifying a novel function for that protein.

Protein-Drug Interactions

Protein microarrays also have great potential for drug discovery and the identification of drug targets. Because the binding profile of a drug of interest can be simultaneously obtained across an entire proteome using this approach, the specificity or side effects of a drug can be monitored. This information should also provide important clues about how to improve drug design (19). To demonstrate that protein microarrays can be used to identify drug targets, Huang and coworkers (20) probed yeast proteome chips with biotinylated small-molecule inhibitors of rapamycin (SMIRs) to find genetic modifiers of the target of rapamycin (TOR) signaling network. They identified candidate drug targets of the SMIRs and validated a previously unknown protein as the bona fide target of the SMIRs. Interestingly, in an independent study, Claudio De Virgilio and colleagues (21) also identified the same protein in the TOR signaling pathway using a different approach.

Protein (Domain)-Peptide Interactions

In a most recent report, Jones and colleagues (3) demonstrated that they could measure quantitative interactions between proteins and peptides in an array format. They cloned, expressed, and purified almost all the human Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains. The total 159 proteins were then printed on the aldehyde-modified glass substrates, and 61 peptides representing physiological sites of tyrosine phosphorylation on the four ErbB receptors were incubated with the protein chips. For quantitative measurement, eight concentrations of each peptide, ranging from 10 nM to 5 μ M, were used in the assay, allowing the binding affinity of each peptide to be measured. With this microarray, 43 of the 65 previously reported interactions were detected, and 116 new interactions were identified. Also, ErbB1 and ErbB2 were found to become more promiscuous with increasing concentration, whereas ErbB3 did not. Because ErbB1 and ErbB2 are

Figure 3. A sandwich assay format. A multivalent antigen is first caught by a capture antibody immobilized on the surface and then detected by a detection antibody. The label is usually tagged on the detection antibody and can be further amplified.

Figure 4. Examples of different assays on functional protein chips. Different types of biochemical assays were carried out on chips, including assays of (A) protein-protein, (B) protein-lipid, (C) protein-DNA, (D) protein-drug, (H) protein-small molecule, and (F) protein-antibody interactions. The chips can also be used to monitor immune responses in patients (G) and posttranslational modifications of proteins, such as phosphorylation (E). These assays achieved high signal-to-noise ratios and were very informative for elucidating the function of previously uncharacterized genes.

overexpressed in many human cancers, the authors suggested that this potential for increased promiscuity might contribute to the oncogenic potential of receptor tyrosine kinases.

Identification of Kinase Substrates on Protein Chips

Since phosphorylation is known to be involved in almost every aspect of cell processes, identification of the downstream substrates of protein kinases is a critical step toward understanding the effects of phosphorylation on protein functions. To demonstrate that the protein chip approach is suitable for such investigations, Zhu and coworkers (22) first analyzed the substrate specificity of 119 yeast kinases on 17 different substrates using nanowell protein chips. Recently, as an extension of the same idea but on a much larger scale, the so-called "Phosphorylome Project" was tested using the yeast proteome microarrays (23). The goal was to identify all the potential protein substrates of each yeast kinase. In vitro kinase reactions were carried out on the yeast proteome chips using 87 individually purified kinases/kinase complexes in the presence of [33 P]ATP. Phosphorylation events (4129), involving 1325 different proteins, were identified. To ensure that the signals resulted from phosphorylation events, 5% sodium dodecyl sulfate (SDS) was used to denature proteins on chips to remove signals from binding of kinase proteins or [33 P]ATP. Those phosphorylation results have been assembled into a first-generation, global kinase signaling network in yeast.

Profiling Immune Responses

The microarray-based identification of the autoantigens targeted by autoantibodies during the immune response has considerable potential for use in diagnosis, classification, and prognosis (24). Robinson and colleagues (25) published the first report of the simultaneous analysis of multiple human disease sera via protein microarray. They arrayed 196 distinct biomolecules involved in eight distinct human autoimmune diseases, including proteins, peptides, enzymes complexes, ribonucleoprotein complexes, DNA, and posttranslationally modified antigens, onto glass slides to form the autoantigen

microarrays. These arrays were incubated with patient serum samples as a means of defining the pathogenesis of autoantibody responses in human autoimmune diseases. Recently, Cahill and colleagues (24) constructed a protein array consisting of polypeptides translated from 37,200 random human cDNA clones in *Escherichia coli* and used this array to identify potential autoantigens involved in the pathogenesis of alopecia areata. Eight autoantigens were identified and successfully confirmed by Western blot analysis. Likewise, a human protein chip containing 2413 nonredundant human fusion proteins was constructed for serum profiling and antibody screening by the same group (26).

Zhu and coworkers (27) also fabricated protein chips that allowed them to rapidly and sensitively distinguish the immune responses of severe acute respiratory syndrome (SARS)-infected and healthy people. These protein chips harbored all the SARS-coronavirus (CoV) proteins as well as proteins from five additional corona viruses that can infect humans (HCoV-229E and HCoV-OC43), cows [bovine corona-

virus (BCV)], cats [feline infectious peritonitis virus (FIPV)], and mice [mouse hepatitis coronavirus (MHVA59)]. The presence of human immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies against SARS-CoV was detected on the chips with labeled anti-human IgG and IgM antibodies. Sera from patients could quickly be clustered as SARS positive or SARS negative on the basis of the serum-probing signals. Comparison to other methods [e.g., enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA)] indicated that the origin of 94% of the sera could be correctly predicted on the microarrays. The chip-based assay was at least 100-fold more sensitive than the ELISA/IFA assays and required a smaller amount of sample.

Conclusion

Protein microarray technology has been shown to be a useful tool for multiplexed detection and proteomics studies. Femtomolar sensitivity has been achieved in analytical protein microarrays, and the number of applications of functional protein microarrays has grown dramatically. It appears that protein microarrays will prove to be one of the most powerful tools in the field of diagnostics and high-throughput biology. Improvements in our ability to generate large sets of high-quality proteins and antibodies or their mimetics will play a key role in quantitative analysis and promote the extension of this technology to other model organisms.

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