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PROTEIN ARRAYS, AN EXCELLENT TOOL IN BIOMEDICAL RESEARCH

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TABLE OF CONTENTS

1. ABSTRACT

Although excellent progress has been made in simultaneous analysis of the whole genome, great challenge remains in the highly paralleled analysis of proteome. The development of protein arrays represents a major technology advance in the proteomics field. Advances in protein array technology include chemical surfaces, capture reagents, detection methods and arraying devices. Protein arrays can be classified into different groups. Potential applications of protein arrays include: 1) measurement of protein expression; 2) identification of interactions between protein-protein, protein-DNA and protein-small molecule; 3) analysis of protein modifications; and 4) determination of antibodies. Such protein array technology holds great promise in biomedical research and clinical applications.

2. INTRODUCTION

As complete genome sequencing of human and other organisms has been accomplished (1-7), the challenge remains to characterize the protein expression and function of proteomes, which holds the key to conquering diseases and uncovering the mysteries of life. Since the discovery of powerful DNA chip and cDNA microarray technology, we now can detect almost entirely whole genomic expression patterns (8-10). However, the technique, which can detect all proteins expressed by a genome, has not yet been perfected, although significant progress has been made. Indeed, protein array technology has contributed significantly to the advancement of whole proteome detection. Furthermore, the function aspects of proteins, such as protein interactions and protein modifications, can only be detected by protein analysis. Therefore, there is a great and urgent need to develop highly paralleled technology such as protein arrays.

The concept of antibody-based microarrays to detect multiple protein expression was proposed in mid- to late 1980 by Drs. Ekin and Chu (11). They proved mathematically that antibody-based arrays are able to determine simultaneously multiple protein levels with high sensitivity (11, 12, 12, 13). Since then, several groups have attempted to establish an antibody-based array assay to simultaneously determine multiple protein expression. Among them, Dr. Silzel et. al. demonstrated that multiple

IgG subclasses could be detected by fluorescence imaging (14). Researchers at Genometrix further demonstrated the possibility of detecting multiple IgGs in a high-throughput format (15). However, the progress of protein array technology has been slow and largely awkward due to past difficulty with high background and low sensitivity. Recently, protein array technologies have been developing rapidly and new technology is emerging at an unprecedented rate. The field of protein arrays is experiencing dynamic and competitive growth. This review will examine the basic concept of protein array technology with particular emphasis on practical applications of protein arrays.

3. A GIANT STEP AND DAUNTING TASK: FROM DNA TO PROTEIN

DNA array technology has been gradually becoming a routine experimental procedure in many research institutions and laboratories (16-18). The ability to detect thousands of genes simultaneously has started to revolutionize biomedical research. Although DNA microarrays are a powerful tool, it can only detect DNA and mRNA. However, proteins do almost all the work of cells. Since proteins are regulated at multiple levels, mRNA levels do not always translate into protein levels. Some evidence suggests that the correlation between the protein and mRNA levels is less than 0.5 (19, 20). In addition, the biological activity of proteins is not always a result of changes in gene expression, rather of modifications such as phosphorylation, arginylation, glycosylation, oxidation, acetylation and methylation, and protein-protein interaction. A well-known example is the activation of the protein signal transduction cascade, which causes a cell to migrate, to die, to differentiate or to proliferate. Those events can take place before any change occurs in DNA/RNA gene expression. In some cases, genomic-based approaches prove more difficult than protein-based detection. One example is to detect protein levels, particularly cytokines, in serum or in tissue culture medium. In this case, detection of protein levels is much easier than detection of mRNA levels since one can simply collect serum or conditioned medium. To detect the presence of an antibody, protein detection may be the only effective way. To identify the potential molecular targets for drug discovery, protein arrays are a more direct approach than DNA-based approaches. Therefore, protein arrays may provide a more realistic hope to the fight against diseases.

Although enthusiasm for protein arrays is increasing in biomedical research community, we are facing many more challenges in the protein world. The behavior and function of proteins are determined by a highly complex structure. First, proteins are easy to denature, and denatured proteins have distinct properties compared to native forms of proteins. Secondly, there is currently no protein amplification system available. In contrast, DNA and RNA can be easily amplified by PCR. Therefore, identification of proteins remains a daunting task. For DNA and RNA, complementary strands can be used to probe and to identify specific DNA. For proteins, antibodies are the most common means of identification. Production of antibodies is often a painstaking process. In addition, proteins are easily degraded. Therefore, it is difficult to handle and manufacture proteins. Furthermore, proteins are more complex, can be modified at multiple levels, and have different “variants. It is estimated from the human genomic sequence that there are approximately 30,000 genes (1, 2). Protein, on the other hand, including modifications and different forms of variants, can number as high as 100,000 to 1 million. How to detect all these different proteins remains a challenge in biomedical research. Finally, the abundance of proteins in different cells and situations can vary up to six orders of magnitude, and not all proteins can be extracted or assayed under a single set of conditions. Therefore, it is much more difficult to deal with proteins than with DNA.

Despite the difficulties and challenges, rapid progress is still being made in analysis of multiple proteins. Since the advent of powerful DNA chip and cDNA microarray technology, detecting whole genomic expression patterns is becoming possible. However, the technique, which detects all proteins (proteome) expressed by a genome has not yet been perfected.

Three major technologies are now available to analyze multiple protein expressions and functions simultaneously. One is a combination of 2-D gel and mass spectrophotometry (Table 1), which is more commonly used in proteomics. Two-D gel to separate proteins based on differences in charge and molecular weight was invented in 1970s. In a typical 2-D gel, several thousand proteins can be detected in one gel. The problem with using 2-D gel over a long time period is how to identify the target spot. Another drawback is reproducibility. It is very difficult to compare two gels. Since the advance of protein technology and mass spectrometry, spots can be relatively easily identified. Spots can be cut out from a gel, digested with trypsin and run on mass spectrometry. Modern mass spectrometry is very powerful and can be very easily to determine a profile of digested proteins. These protein “fingerprints” can be checked against protein and DNA sequence databases. When a matching sequence is found, it can be used to annotate and predict the full amino acid sequence of the protein. If the protein sequence cannot be predicted by fingerprints, mass spectrometry can sequence the peptide and search the database to find a matching protein. Recently, an automated sequencing system has been developed. Automating the process has made proteomics a highly profitable area (21-25). Collecting protein expression data and building databases of proteomics are being undertaken. However, this approach presents several problems. It requires sophisticated devices and significant downstream efforts to identify proteins. Some proteins with particularly low molecular weights cannot be identified using this method. In addition, sensitivity is lower and the detection limit is at ng levels in 2-D gel systems. Unfortunately, many important proteins are expressed at much lower levels than 2-D systems can detect.

Table 1. Comparison of Three Major Technologies of Proteomics

	2D gel/MS	SELDI	Arrays
Instant identification	no	no	Yes
Discovery of new protein	yes	maybe	no
Detection of HMWP	yes	Not optimal	yes
Detection of LMWP	not optimal	yes	yes
Sensitivity	low(ng)	very high (fg)	high(ng fg)
Detection density	high	low	high
Speed	slow	Extreme fast	fast
Cost	very high	high	relative low
Sample volume	large	tiny	Small
Experiment setup	challenge	easy	variable from easy to challenge
Quantitation	not to semi-quantitative	semi-quantitative	semi to quantitative
Major application	profiling	Biomarker discovery	Diagnosis, profiling, protein-protein interaction, drug discovery

Abbreviations: HMWP, high molecular weight protein; LMWP, low molecular weight protein.

Another promising technological advancement is called SELDI protein chip (surface enhanced laser desorption/ionization protein chip) (Table 1) (26). This system is based on an aluminum chip spotted either with chemicals or with antibodies that bind to the protein. The surface of chip is coated with chemicals or other materials. Tiny amounts of samples, e.g. a couple of µl, are applied to the surface of the chip. Some proteins will bind to the surface; unbound proteins will be washed away. A chemical energy absorbing molecule (EAM) solution is then applied and allowed to dry, during which time minute crystals form on the chip. These crystals contain the EAM and the protein(s) of interest. The proteins now can be read by a protein chip reader. When the laser beam hits the proteins embedded in the EAM crystals, the proteins will be desorbed and ionized. The released ions then fly through a vacuum tube towards the ion detector. Finally, the ionized proteins are detected and an accurate mass is determined based upon the time of flight. The system is easy to operate, relatively cheap, and has a high throughput. Therefore, this approach is gradually becoming a popular tool in the discovery of biomarkers in different diseases (27-31). Unfortunately, the system cannot identify the identity of captured proteins, and only a handful of proteins can be profiled at same time. In addition, it is difficult to detect high molecular weight proteins in SELDI. This technology is primarily utilized for detection of patterns in protein profiling. Therefore, developing a new approach to profiling protein expression and function is quite urgent.

An alternative approach that eliminates the substantial downstream effort in the identification of proteins is to apply an array format to detect protein expression (Table 1). Arrays have the advantage of being scalable, flexible and easy to use. The nature of arrays allows a high-throughput screening using robotic, imaging, or analytical methods. The major disadvantage for protein arrays is the system cannot be used to discover novel proteins.

Other technologies, such as high performance liquid chromatography (HPLC) (32, 33), capillary electrophoresis (CE) (34), and bead-based arrays (35-38), also are starting to be applied in multiple protein detection.

4. PROTEIN ARRAY TECHNOLOGY

Many factors will affect the success of the development of protein array systems. Major among them are supports and chemical surfaces, capture reagents, detection approaches, and printing methods.

4.1. Supports and chemical surfaces

Three major supports are currently available: glass slides, membranes, and plates. Glass slides are suitable for high throughput and have low background for fluorescence detection. The major problem with glass slides is the poor protein binding capacity. To overcome this problem, glass surfaces can be treated with aminosilane, poly-lysine, or aldehyde surface activation (39). We found that hydrogel slides are particularly suitable for protein arrays with high binding capacity and low background (40, 41). Such hydrogel slides can be used for a variety of sample sources such as cell lysate, tissue lysate and body fluids. Many companies and academic labs are developing different sophisticated surface chemistries for protein array applications.

Membranes, on the other hand, have been widely used in Western blotting assays and have a high binding capacity. Proteins can bind to membranes through non-covalent interactions with hydrophobic (nitrocellulose and polystyrene) or positively charged (poly-lysine and aminosilane) surfaces. Membranes usually are inexpensive and are particularly useful for macroarrays. We routinely use membranes as a macroarray format for testing new concepts at the proof of principle stage and for developing new assays (40, 42-44). The advantage of using membranes as a macroarray format is the procedure can be done in any lab without sophisticated equipment. Membranes can also be adhered to glass slides. The problem with membranes is the high background, especially when tissue lysate and cell lysate are used. Fabrication of microarrays consisting of G protein-coupled receptors (GPCRs) on membrane surfaces coated with gamma-aminopropylsilane (GAPS) using direct pin-printing has been described (45). Another support is tissue culture plates, usually 96 well plates (15, 46). The advantage of this format is that experiments can be performed using the existing automatic liquid handle system. The major limitation is it is difficult to create a high-density array system.

Most supports are good for antibody arrays to certain degree. However, unlike antibodies, antigens have considerable distinct surface binding properties. To achieve uniform binding of all antigens to surfaces, specific biomolecular interactions such as streptavidine-biotin (47-

50), his-tag – nickel-chelates (51), antibody-antigen (44) or formation of stable complexes between phenylboronic acid labeled analytes and salicylhydroxamic modified surfaces (Versalinx ™ technology, Prolinx Inc., Bothell, WA, USA), have been introduced in immobilization of proteins onto solid supports. The major concern for these approaches is the lack of high labeling efficiency for every protein and the increased complexity by additional steps. Only the best system will survive in the future for practical applications.

4.2. Capture reagents

To successfully develop a highly specific and sensitive protein array technology, capture reagents are the key. The ideal capture reagents should 1) have high specific and affinity to the target molecules, 2) be easy to produce and handle, 3) have a large library of molecules available for construction of very high density of arrays, and 4) if desired, have a signal which can be amplified. Currently, no capture reagents meet all these requirements.

The most common capture reagent is antibody. Currently, there are thousands of antibodies available commercially. Some antibodies have high affinity and specificity to their targets. Antibodies can be generated in three different ways: polyclonal antibodies, monoclonal antibodies and phase-antibody display. Polyclonal antibodies usually have high affinity and are easy to produce; however, the supply is limited and is difficult to set up standardization of the assay technique for large quantities of samples for future applications. Monoclonal antibodies, on the other hand, are in unlimited supply; therefore, it is possible to standardize both reagents and assay techniques. However, the production of monoclonal antibodies is often time consuming, labor intensive and expensive. Phase antibody display and ribosome display are potential effective alternatives to producing antibodies (52). It has the potential to generate entirely synthetic combinatorial antibody libraries against nearly any target (53-55). The selection of the target molecules for corresponding antibodies from those libraries still remains a great challenge to the biomedical community since the availability of pure antigens. To overcome this problem, Aldveron (Farco, North Dakota, www.aldevron.com) created a new approach to producing polyclonal antibodies from cDNA expression plasmids, thereby avoiding the need of recombinant or synthetic proteins (56-58). Other advantages of this approach include high affinity antibodies that recognize native forms of proteins, and greater antibody response than conventional genetic immunization protocols. This approach can also be adapted to generate monoclonal antibodies (59), although conventional approaches still must be applied. Since polyclonal antibodies can be effectively generated from synthetic peptides, how well this system will serve the research community still remains to be seen.

Antibodies, the most popular class of molecules providing traditional molecular recognition needs, are not the only capture agents available. Aptamers, oligonucleotides that bind to proteins with high specificity and affinity, can also be used. Such oligonucleotides can be isolated using a procedure called SELEX (systematic evolution of ligands by exponential enrichment). Although isolations of aptamers can be a complicated process, the SELEX protocol has been successfully automated (60, 61). Aptamers can be synthesized, thereby providing unlimited sources and making standardization procedures easy. They are stable and easy to handle. The use of photo-cross-linkable aptamers allows the covalent attachment of aptamers to their cognate proteins with very low backgrounds from other proteins in body fluids. Furthermore, proteins can be directly detected using any reagent or procedure, which distinguishes functional groups of amino acids from those of nucleic acids (and the solid supports). Therefore, aptamers are very attractive capture reagents for protein array applications (62). SomaLogic (Boulder, CO, www.somalogic.com) is developing aptamer arrays for protein detection.

Peptides are another attractive candidate for capture reagents in protein arrays because they combine the advantages of small molecules and proteins. Highly diverse peptide libraries can be generated either biologically or synthetically (52, 63). The protein-binding peptides then can be isolated by screening such libraries. The advantage is that the peptides can be made synthetically in large quantities in a relatively inexpensive manner after the protein-binding peptides have been identified. However, affinity is usually low. Efforts are also being made in using peptide-like oligomers (64-66). Such materials are impervious to proteases, and their synthesis can be simpler and cheaper than that of peptides. Low affinity to their target proteins is still a major concern for these reagents. To increase the affinity of peptides to proteins, approaches to search peptides from a library displayed on the surface of a protein have been developed (67). By incorporating a peptide into a surface-exposed protein loop, the degree of freedom of a peptide is reduced, but the affinity is increased (68). The protein-binding peptide can also be screened using yeast two-hybrid system *in vitro* (69) or *in vivo* (70).

The most robust way to prepare capture reagents is small molecule ligands (71). Small molecule ligands can be synthesized chemically, thus making supply of protein-binding ligands much simpler and standardization possible. Small molecular ligands can be identified through combinatorial library methods (72). Many small molecules have been identified as therapeutic agents, but can also be used as protein ligands. The problem with small molecular ligands is their low affinity.

Purified or recombinant proteins are another type of capture reagent, particularly useful in functional analysis of proteins such as protein-protein interaction, posttranslational modifications, protein-small molecule interaction and autoantibodies. Recombinant proteins can be purified from bacterial *in vitro* expression systems as a fusion protein. The most common fusion protein expression system is protein fused with glutathione S-transferase (GST) or Hisx6 or both. The tags are then used to purify the expressed fusion proteins. Zhu *et al.* (51) established a miniaturized system to purify almost an entire yeast

proteome and created microarrays of 5,800 yeast recombinant proteins. Such a recombinant system is powerful in the generation of many recombinant proteins as capture reagents for protein arrays. However, the labor intense procedure limits its broad application. To eliminate the cloning and purification steps, He *et al.* (73) developed a new procedure termed PISA (protein *in situ* array). Using this procedure, protein arrays can be generated directly from PCR-generated DNA coupled with cell-free *in vitro* translation and simultaneous *in situ* immobilization of the generated proteins on a surface. Such an approach allows the one-step-generation of protein arrays. If PISA is proved to be an efficient and reliable method, it will be very useful for generating high-density protein arrays containing toxic proteins and membrane-bound proteins.

Another strategy that produces large numbers of proteins for protein arrays is PROfusion technology (Phylos, Inc, www. Phylos.com, Lexington, MA). By covalently attaching a puromycin to the 3' end of the mRNA, the PROfusion system contains a large pool of fusion molecules consisting of a protein linked to its own messenger RNA. Using PROfusion technology, Phylos created libraries of up to 100 trillion proteins. Such libraries should provide excellent resources to isolate appropriate capture proteins with known identity. Since the advance of protein separation technology, investigators can now start to purify all proteins from a cell lysate with the goal creating a protein array system using purified proteins (74).

4.3. Detection methods

The most sensitive approach to detecting proteins is chemiluminescence, since the signal is amplified by turnover of the enzyme. Chemiluminescence detection is used to detect proteins on filter arrays (40, 42-44, 75, 76), glass arrays (77) and tissue culture plates (15). Initial study suggests that chemiluminescence detection can be quite quantitative and have dynamic ranges (15, 43, 44). The signals can be recorded in x-ray films; therefore, no major equipment is required. The major limitations of chemiluminescence are the short window to detect a signal and the availability of only one signal channel.

The most common approach to detecting proteins is fluorescence. Fluorescence can be directly labeled into proteins (78) or conjugated into other detection molecule such as streptavidin (40, 79). Fluorescence detection offers multiple channel detection capacity and long-lasting signals. The sensitivity of fluorescence detection is usually lower than chemiluminescence. Since there is low auto-fluorescence in glass slides, protein arrays are normally constructed on glass when using fluorescence detection. Low auto-fluorescence membranes have been produced by Schleicher & Schuell (.Schleicher & Schuell Dassel, Germany. www.s-und-s.de). The fluorescent signals were then detected by laser scanner or CCD- cameras. One major concern for directly labeled proteins is the uniform labeling efficiency and the potential alternation of the high structure of labeled proteins.

Isotopic labeling has been used for detecting protein-protein interactions, protein-nucleic acid interactions, and of protein modifications in protein arrays (80, 81). Although isotopically labeled proteins retain native configuration and have very high sensitivity, extract caution for handling with isotope greatly limits it wider application.

Mass spectrometry is being using to detect low-density arrays and is commercially available from Ciphergen (Palo Alto, CA). The system is very powerful for identifying expression patterns from different samples.

Other detection methods may also be used for protein arrays. They include surface plasmon resonance (SPR) (82), planar waveguide technology (83), electrochemical detection (84), quartz crystal microbalance (QCM) (85-87) and microcantilevers (88, 89).

4.4. Arraying devices

Protein arrays can be generated either automatically or manually. Manual arrays are the most convenient and cost-effective way to set up an array system. The arrays can be generated by either manual spot (65, 66, 90) or immuno-dot-blot devise (64, 80). Obvious problems for manual arrays include lack of ability to construct high-density arrays, high consumption of samples and reagents, and possibly lower sensitivity. Therefore, most researchers use an arrayer to print capture reagents onto solid supports. Two types of arrayers, direct contact and noncontact, are currently on the market. Most direct contact arrayers are pin tool based arrayers. These arrayers deposit miniscule amounts of proteins onto a surface by contact. The amounts of proteins deposited can be controlled by the size of the pin; therefore, it is difficult to control the exact amounts of proteins to be deposited onto the surfaces. Noncontact arrayers are mainly ink-jet printing and peizoeletronic robotic dispensed. Protein solutions in cleaned ink cartridges are spotted onto a surface using a commercially available ink jet printer (14, 91). Such a system can be made in the laboratory and produces consistent spots quickly, but some proteins may be denatured or degraded during the deposition process due to high temperature and shearing force. Furthermore, it may be difficult to control the exact amount of protein deposited and it may be cumbersome to deposit many different proteins. To date, peizoeletroic robotic dispensers may be the best system to print protein solutions onto surfaces. The exact amounts of protein solutions are squeezed out onto a surface without touching. Since the volumes can be precisely controlled and no heating is necessary for spotting, such peizo-arrayers are becoming popular in protein arrays. One potential problem for this type of arrayer is background and cross-reaction due to the splash of dropping solutions.

For peptide arrays, peptides can be synthesized on a surface by photolithography (92) or on continuous cellulose supports (93). Protein arrays can also be generated from DNA by cell-free expression and *in situ* immobilization (PISA method) (73). Electrospray deposition (94, 95), scanning probe based lithography (96, 97), soft lithography technologies such as microcontact printing (μCP) (98, 99) and precision glass syringe (100) may open new avenue for construction of protein arrays in the future.

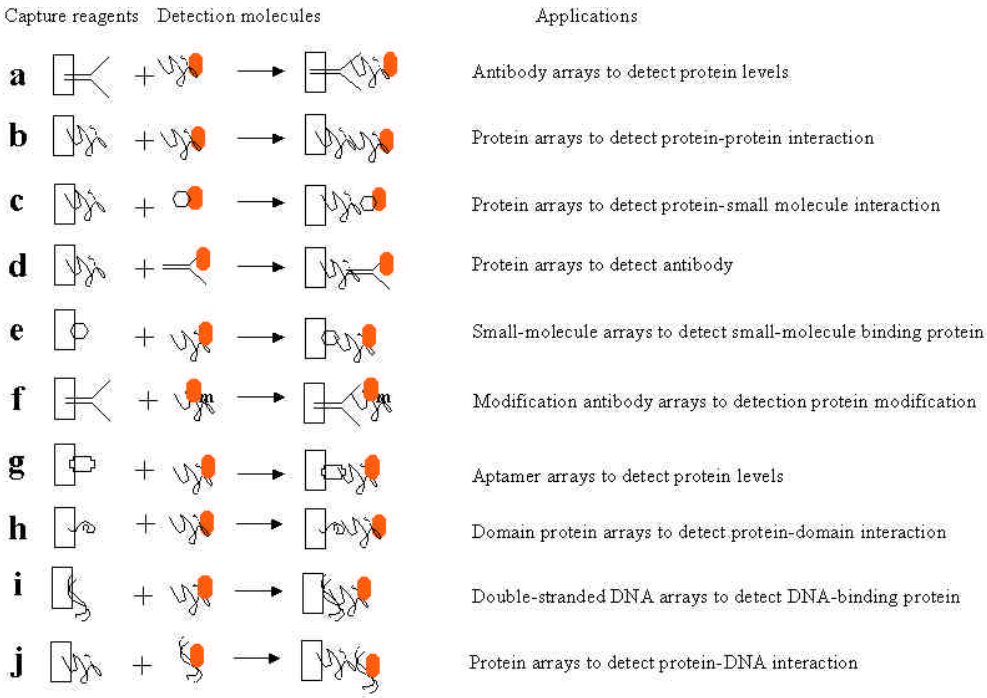

Figure 1. Label-based protein arrays. In these approaches, different classes of molecules, which function as capture reagents, are spotted onto solid support. Corresponding molecules (detection molecules), which bind to capture reagents, are labeled using relevant methods. The labeled detection molecules are incubated with array support. The signals are then detected using detection devices and reflect the presence and/or relative amount of detection molecules.

5. CLASSIFICATION

There is no unified method of classifying protein arrays. Different investigators refer to their systems by different names. Protein arrays can also be classified into different groups.

5.1. Applications

Protein arrays can be classified into two major categories according to their applications: 1) protein function arrays, and 2) protein detection arrays (protein expression arrays) (71). Protein arrays have two major applications. One is to apply protein arrays for highly paralleled detection of protein expression levels, including antigen and antibody in semi-quantitative and quantitative ways. These types of protein arrays are therefore referred to as protein expression arrays. They mainly serve as an analytic tool somewhat analogous to DNA arrays. If protein expression is solely controlled at the transcription level, then DNA arrays may partially substitute for protein arrays. Another application of protein arrays is to study protein-protein interactions, protein modifications, DNA-protein interactions, RNA-protein interactions and small molecule-protein interactions. Since these types of protein arrays primarily detect the biological activities of proteins, they are referred to as protein function arrays. The information obtained from these types of protein arrays cannot be replaced by genomic-based array technology.

5.2. Capture reagents

Several capture reagents are used for protein arrays. The most common capture reagents for protein arrays are antibody and recombinant protein or purified protein. They are referred as antibody arrays and protein arrays, respectively. Peptide arrays (101-103), aptamer arrays (60), and small molecule arrays (104, 105) reflect the specific capture reagents used in protein arrays. To detect autoantibodies, autoantigens are used as capture reagents; therefore, these types of protein arrays are known as autoantigen protein arrays or antigen arrays (106).

5.3. Densities

Protein arrays can be generated using different methods, thereby having different densities. High-density arrays are referred to as protein microarrays or protein chips, and low-density arrays as protein macroarrays.

5.4. Samples

Protein arrays can be used to detect single proteins from different biosources. Therefore, protein arrays can also be named cell lysate arrays, conditioned medium arrays (44), serum arrays (44) and reversed phase protein arrays (107).

6. APPLICATIONS

Like DNA microarrays, protein arrays can be used to determine differential expression. Besides that, protein arrays can also be used to determine protein-protein interactions, protein-modifications, DNA-protein interactions, small molecule-protein interactions and antibody detection. The applications of protein array

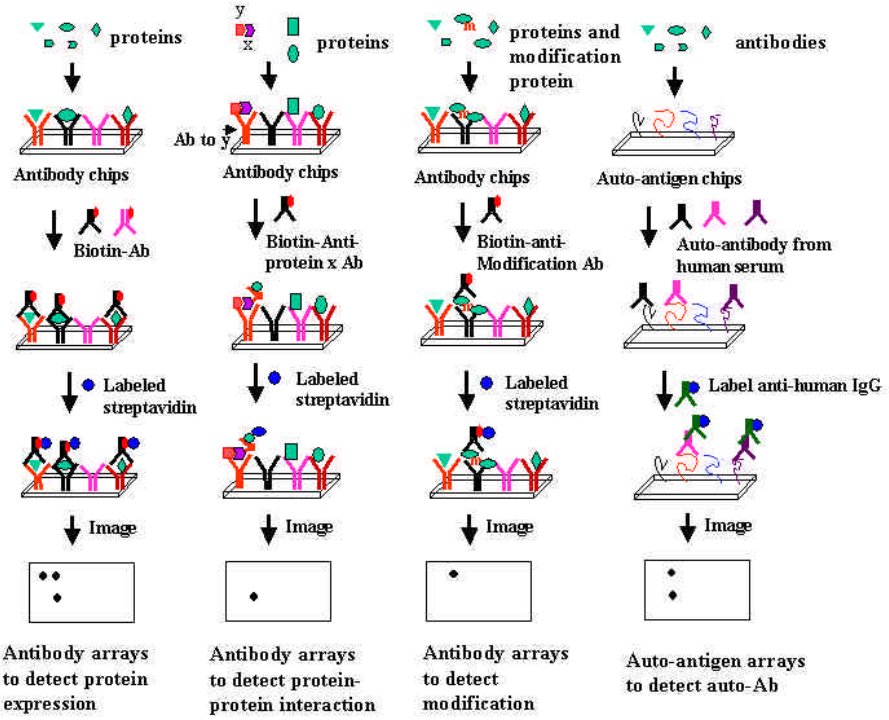

Figure 2. ELISA-based protein arrays. In these approaches, array chips spotted with different capture reagents are incubated with samples. Molecules bound to array chips are detected by labeled detection reagents. X and y represent a pair of interacting molecules. M stands for modification.

technology are rapidly growing. Unlike DNA microarrays, many different approaches have been developed for protein arrays. Those approaches can be classified into two main types: label-based approaches (Figure 1) and ELISA-based approaches (Figure 2). The principle of the label-based approach is similar to DNA arrays. The samples are labeled with detection dye and applied to array supports. After removing the unbound molecules, the proteins captured on the surface are detected from the labeled dye. The advantages of this system are the possibility to create very high-density arrays, only one capture molecule is required and the system is simple and straightforward. The problems inherent in this approach are lack of uniform labeling efficiency for every molecule, and potential alteration of high structure due to conjugation of detection molecules. On the other hand, the ELISA-based approach relies on two binding molecules, one of which functions as capture reagent and the other of which serves as detection reagent. Samples are incubated with array support. The molecules retained on the surface of the array support will be detected by a detection reagent, which binds to the same molecule as capture molecules but in different epitopes. The advantages of this system are the maintenance of high structure of proteins to be detected, potential quantitative measures, and high specificity. The drawbacks are it is impossible in some cases to create a high-density array system, and it is sometimes difficult to find matched pair reagents binding the same molecules in different epitopes.

6.1. Differential protein expression

Cell physiology and pathology are very complex phenomena. Every biological process involves many genes. Thus, there is a growing consensus that global analysis of gene expression is vital for us to understand cell functions, the maintenance of health state of human beings, and the development of diseases. One of the major applications of protein arrays is to determine protein expression.

Several approaches have been developed. Sandwich ELISA is popular in the detection of cytokine expression as shown in Fig 2a (15, 42-44, 79, 108). In this approach, two antibodies are required for detection of a single protein. One antibody serves as capture reagent and is spotted onto a solid surface. Another antibody functions as detection reagent and is usually labeled with biotin. The advantages of this approach are that the experiment is easy to perform and quantitative measurement is possible. The disadvantages are it is difficult to develop high-density arrays since it is almost impossible to mix thousands of capture antibodies together, and it requires two antibodies recognizing two different epitopes.

We have created both macroarray and microarray platforms to simultaneously measure cytokine expression in ELISA-based protein arrays (40, 43, 44). The macroarray format allows the design of arrays in a simple, inexpensive and flexible way. Because no sophisticated equipment is required in the entire process, the system can be accessed by a wider research community. The microarray format allows the development of a high-throughput approach to screen large quantities of samples with very efficient reagents. The system also has high specificity and sensitivity to detect physiological levels of those factors. Using these systems, we have successfully identified MCP-1 as a potential target involved in cx43-mediated tumor suppression (40) and several chemokines as molecular targets of antioxidant vitamin E (109).

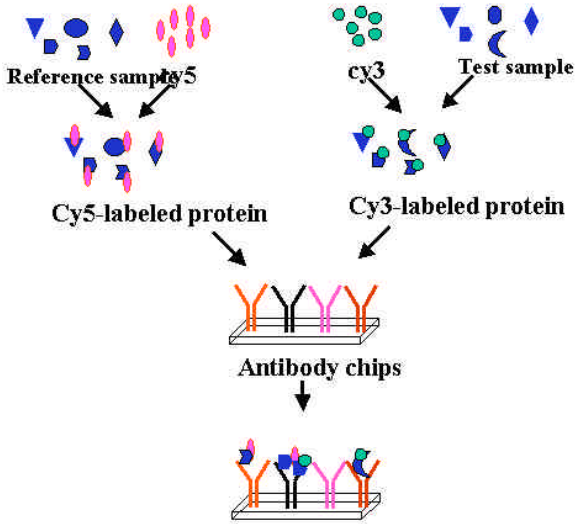

Figure 3. Schematic representation of two color protein arrays. One sample is labeled with cy3. Another sample is labeled with cy5. Both samples are then mixed together and incubated to protein chip. Signals are detected by laser scanner with cy3 and cy5 channels. The fluorescence ratio between the color channels reflects the relative protein concentrations between the two samples.

Another common method of detecting protein expression in protein arrays is to directly label proteins with either fluorescent dyes (14, 39, 78, 110) or biotin (111) as shown in Figure 1a. Since multiple fluorescent dyes are available commercially, two-color detection can be used to detect the expression of proteins from a test sample and reference sample on the same slide as shown in Figure 3. This approach avoids the variability attributable to spot size and amount of antibody deposited and incubation condition, making comparisons across microarrays more valid. Two major concerns regarding this approach include potential change of high structure and lack of uniform labeling. Using this approach, Sreekumar *et al.* (110) studied the signal pathways involved in radiation-induced apoptosis. They observed that radiation treatment down-regulated carcinoembryonic antigen.

Mass spectrometry has been used to detect low-density arrays of captured proteins and is available commercially through the company Ciphergen (Palo Alto, CA). Tiny amounts of crude samples are applied to protein chips, which have been coated with an array of various capture reagents. After removing unbound proteins, the bound proteins are then laser-ablated and analyzed by mass spectrometry. The expression profiles are generated from different samples. This approach has been proven as fast, easy and reproducible, and is gradually gaining attention in biomarker discovery (112-115). By combination of surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) and artificial-intelligence-based informatics algorithms, Petricion *et al.* have discovered a small set of key protein values that discriminate normal from ovarian cancer patients. Their approach yielded a sensitivity of 100% (95% CI 93--100), specificity of 95% (87--99), and positive predictive value of 94% (84--99). These findings indicate that SELDI is a potentially powerful to in biomarker discovery in the early detection of cancer. Similar efforts have been made in the identification of early biomarkers in other cancers such as breast cancer, prostate cancer and lung cancer (115).

6.2. Protein-protein interaction

It is generally appreciated that most biological events are mediated by protein-protein interactions comprised of from several to almost a hundred different proteins (116). Elucidation of protein-protein interaction maps will not only have biological significance, but also a great impact on drug discovery. The key for this application is the availability of recombinant proteins or purified proteins. The recombinant or purified proteins are then spotted onto the array support. Other purified proteins are then applied to the array slides. The presence of proteins can be detected and indicated as interactions take place as shown in Figure 1b. A breakthrough for protein-protein proteomics has been achieved by Zhu *et al.* (51), who successfully expressed all 5,800 open reading frames of yeast and created a protein chip containing all 5,800 recombinant proteins in array formats. Using this proteome chip to screen the calcineurine binding proteins, Zhu *et al.* identified an additional 33 new potential binding partners of calmodulin, which contain a potential binding motif, in addition to 113 known Camkinases and 114 calcineurins (51). This is the first genome-wide analysis of calcineurin interacting proteins. Such an approach should allow us to construct whole genome-wide protein-protein interacting maps. However, the interaction only reflects the *in vitro* protein-protein interaction. Many factors, such as modifications, ions and cofactors, can influence protein-protein interactions *in vivo.* Furthermore, the purity of recombinant protein may be another major concern. In addition, the labeled protein may change their higher configurations, thereby affecting the binding specificity and affinity of interacting proteins. Finally, many membrane-bound proteins and toxic proteins are difficult to express in the bacterial expression system.

The input protein can be expressed as green fluorescent fusion protein, therefore no *in vitro* labeling is necessary (117). However, such an approach still presents the same concerns as the *in vitro* labeling approach.

Some of the limitations of the above approach may be overcome by antibody arrays (118). The principle for detection of protein-protein interactions can be illustrated in Fig.2b. This approach has been used to study the TNF-alpha signal transduction pathway and identify stat1 as a component of the TNFR1-TRADD signaling complex. Two major advantages if this system are that no pure protein is required and the protein-protein interaction is more likely to resemble *in vivo* situations. The concern is that both capture and detection antibodies must recognize different epitopes from the protein-protein interacting sites.

Different domains or motifs can also be used to generate protein arrays to identify the potential protein interacting domains or motifs (119). Protein domains mediate protein-protein interactions through binding to

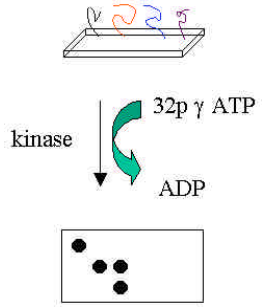

Figure 4. Kinase protein arrays. Protein kinase substrates are immobilized onto solid support in array format. The array chips are then incubated with protein kinase in the presence of ^{32}P-ATP. ^{32}P will be incorporated into kinase substrates by corresponding protein kinase.

short peptide motifs in their corresponding ligands. These peptide recognition modules are critical for the assembly of multiprotein complexes. Such protein-domain interactions can be identified by protein array technology as shown in Figure 1h. Espejo *et al.* have generated a protein-domain chip containing arrayed protein-interacting modules such as WW, SH3, SH2, 14.3.3, FHA, PDZ, PH and FF domains. To identify the potential protein interacting domain, fusion proteins were then incubated with protein-domain chips and the domain-bound proteins were detected on the chips with a specific antibody. Using this approach, they have confirmed the domain binding profile of the signaling molecule, Sam68, and have identified a new binding profile for the core snRNP protein, SmB. This protein-domain chip not only identifies potential binding partners for proteins, but also promises to recognize qualitative differences in protein ligands (caused by post-translational modification). Such protein-domain chips are easily generated and will have broad applications, particularly in signal transduction.

6.3. Protein modifications

Protein modifications have significant impact on protein function, particularly on those proteins involved in signal pathway and cell cycle control. Several strategies can be adapted to detect modifications of proteins.

One approach is to use labeled substrates which will be incorporated into proteins as modifications take place, e.g. using labeled ATP to detect phosphorylation modification as shown in Figure 4. Zhu *et al.* (120) constructed an array containing different types of substrates. The arrays were then incubated with purified kinase in the presence of ATP. The kinase's substrates were suggested by the incorporation of radioactive ATP in the substrates. Using this approach, novel activities of individual kinases were identified. Sequence comparisons of enzymes, which phosphorylate tyrosine residues, revealed that they often share common amino acid residues around their catalytic regions. This approach has been used by many other investigators to study enzyme-substrate interactions (77, 80).

Antibodies against the modified forms of protein can also be used to generate protein arrays. In this approach, samples are incubated with antibody arrays and the modified proteins are detected by antibodies against specific modifications as shown in Fig.2f. Lesaicherre *et al.* (102) developed a novel fluorescence-based approach for quantitative detection of peptide phosphorylation on chips using fluorescent-labeled anti-phosphoserine and anti-phosphotyrosine antibodies. This method is sensitive, specific and fast, and may find wider uses in high-throughput kinase screenings.

Benjamin *et al.* (121) developed a novel peptide chip by the Diels-Alder-mediated immobilization of the kinase substrate on a self-assembled monolayer of alkanethiolates on gold. They successfully demonstrated that their peptide chip can be used to identify phosphorylation and inhibitors of kinase.

Other strategies are to use modification specific antibodies, e.g. antibodies against tyrosine phosphorylation, serine phosphorylation, acetylation. The principle of this approach is illustrated in Figure 2c. Such an approach is easy to perform, but detection depends entirely on the antibodies against specific modifications. Currently only limited numbers of good antibodies against modified proteins are available.

6.4. Protein-DNA interaction

The interaction between DNA and proteins can be detected through printing proteins or double-strand DNA onto solid surfaces as shown in Figure 1i and Figure 1j. Zhu and coworkers (51) overexpressed and purified 5,800 yeast proteins as GST/HisX6-fusion products. All these proteins were printed onto nickel-coated slides at high spatial density. Labeled, double stranded DNA can be used as a probe to screen this type of protein chip. The interacting proteins can be identified rapidly. On the other hand, Bulyk *et al.* (122) created microarrays of double stranded oligonucleotides, which could be useful for the characterization and identification of DNA binding proteins such as transcription factors.

Both approaches have the ability to identify DNA-binding proteins globally and to find new cis- or trans-elements. The major limitation is that only a few, if not a single, labeled protein or DNA can be used in the screening. To profile multiple transcription factors simultaneously, Panomics company (Redwood city, CA, www.panomics.com) developed trans-signal protein/DNA arrays. In this system, as shown in Figure 5, nuclear extracts are labeled with magnetic beads and incubated with multiple consensus transcription factor binding sequences labeled with biotin. The bound double stranded oligonucleotides are then precipitated with protein and used as probes to hybridize the same double-stranded oligonucleotides spotted onto membranes. Using such a system, more than 90 transcription factors can be identified simultaneously.

6.5. Small molecule-protein interaction

Identification of small molecular binding proteins is a major approach in drug discovery. Protein arrays will significantly speed up this process. Several different approaches have been used to create protein arrays for drug discovery.

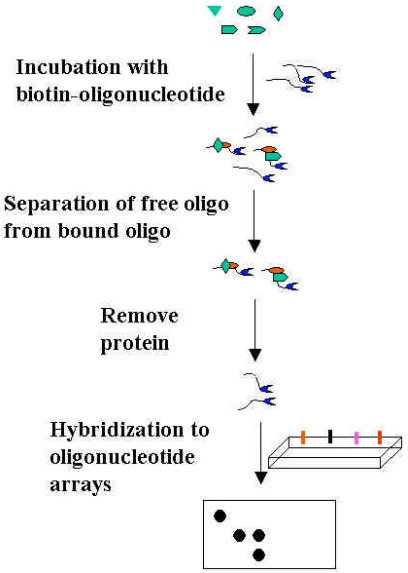

Figure 5. Schematic flow of protein/DNA array. Nuclear extracts prepared from cells or tissues are incubated with a cocktail of label-oligonucleotides such as biotin-oligonucleotides. After separation of free oligonucleotides from DNA-protein complex, the oligonucleotides bound to transcription factors are used as probe to hybridize the oligonucleotide array membranes. Signals reflect the corresponding DNA binding proteins bound to the oligonucleotide.

One obvious process is to immobilize small molecules onto chips then incubate them with labeled target proteins. The presence of proteins in particular spots indicates the potential target molecules as shown in Fig.1e. This technology enables parallel high-throughput screening of small molecular protein or ligand-receptor interactions, but requires only very small quantities of the sample, which could improve screening for active substances in the pharmaceutical industry. Using this approach, Ducruet *et al.* identified FY3-alphaalpha09 and FY21-alphaalpha09, new Cdc25 dual specificity phosphatase inhibitors in targeted small molecule arrays (104).

Kuruvilla *et al.* (105), using a similar approach, identified uretupamine, which specifically activates a glucose-sensitive transcriptional pathway downstream of Ure2p. These results demonstrated that diversity-oriented synthesis and small molecule microarrays can be used to identify small molecules that bind to a protein of interest, and that these small molecules can regulate specific functions of the protein.

Small molecule binding proteins can be identified using labeled small molecules to screen the protein array slides. MacBeath *et al.* demonstrated that this approach can be used to sensitively identify small molecule binding proteins in a high-throughput manner (39).

The potential problems for both approaches are that the data only can tell us which small molecules may bind to certain proteins, or which proteins may bind to certain small molecules. However, no information on the biological impact due to such interactions can be obtained.

To identify the potential biological impact of such interactions between small molecules and proteins, Arenkov *et al.* (77) developed gel-immobilized compounds on a chip (MAGIChip) to screen inhibitors of enzymatic reactions. They immobilized a small set of active enzymes within a hydrophilic gel matrix. Enzyme substrates were then added to the chip and the enzyme reaction took place. The end products, if the substrates were cleaved by enzymes, were precipitated only within the tiny pads on the chips containing active enzymes. Enzyme activity was blocked by addition of enzyme inhibitors to the system. As the screening for enzyme activity and enzyme inhibitors can be performed in parallel, such assays could be useful for high-throughput screening for enzyme inhibitors.

6.6. Antibody detection

Another application of protein arrays is to detect antibodies or map antibody-antigen interaction domains. The similarity of different antibodies from the same species in mRNA sequence makes it difficult to be detected by a RNA-based technology, such as Northern blot, RT-PCR and cDNA microarrays. Protein-based assays may be the most effective way to detect antibodies. Tomlinson's group developed an antibody-array technique for high-throughput screening of recombinant antibodies (123, 124). This technology can be used to isolate antibodies against impure proteins and complex antigens. We also showed that multiple antibodies could be simultaneously detected using protein arrays with high specificity and sensitivity (43). The principle of this approach is shown in Figure 2d. Since only one detection antibody against the species-specific immunoglobulin is needed, this system is much easier and simpler than the protein detection system.

As discuss below, the detection of autoantibodies using protein array technology will have a profound impact on clinical diagnosis and patient care management.

6.7. Carbohydrate detection

Carbohydrate is another type of macromolecule that is involved in many aspects of cell physiology and pathology. The complexity of its structure greatly limits our understanding of carbohydrates. Recently, an array method was developed by Houseman and Mrksich in which carbohydrate chips were prepared by a novel procedure that allows the covalent attachment of carbohydrate-diene conjugates to a specially engineered monolayer surface (125, 126). These carbohydrate chips can be used to analyze protein-carbohydrate interactions and to characterize the substrate specificity of a carbohydrate-modifying enzyme. The study paves the way for the development of highly complex carbohydrate arrays.

6.8. Diagnosis

Protein expression arrays can be used to examine multiple tumor markers at the same time. This will allow us to develop better diagnostic tools for cancer. The sensitivity of protein array systems will provide a broad understanding of disease changes, particularly infectious and

immunological diseases, thereby allowing us to develop better diagnostic tools. Current diagnostic methodology can only measure changes in one antibody at a time. This greatly limits accurate diagnoses. Simultaneous detection of multiple antibodies will provide better diagnosis and greatly reduce costs.

One means of using protein arrays for clinical diagnosis is detection of autoantibodies. Robinson's group has developed autoantibody arrays to profile autoantibodies for diagnosis of autoimmune diseases (106), such as myasthenia gravis, Grave's disease and SLE. Multiple autoantibodies are present in those diseases. Profiling of multiple autoantibodies will have diagnostic utility (127). Autoantigen microarrays are produced by attaching hundreds of proteins, peptides and other biomolecules to the surface of glass slides using a robotic arrayer. Arrays are then incubated with patient serum, and spectrally resolvable fluorescent labels are used to detect autoantibody binding to specific autoantigens on the array. Autoantigen microarrays represent a powerful tool to study the specificity and pathogenesis of autoantibody responses, and to identify and define relevant autoantigens in human autoimmune diseases. Autoantibody arrays can also be used to monitor epitope spreading (127-129). Epitope spreading of the autoantibody response may represent a common harbinger of more severe and progressive autoimmunity, providing the physician with valuable information for patient care management. Autoantibody isotype usage, autoantibody discovery and characterization, and guiding for antigen-specific therapy are among several potential applications of autoantibody arrays.

Another application of protein array technology is to detect multiple bacteria and viruses. Rowe *et al.* developed a fluorescence-based array immunosensor for the simultaneous detection of clinical analytes using a sandwich format (130). Such array immunosensors were effective for detection and measurement of targets like bacteria and protein toxins at physiological levels. This planar waveguide system is an attractive choice to perform highly sensitive, miniaturized and parallelized immunoarrays.

Bacterial and viral infections can also be diagnosed by detection of antibodies against bacterial and viral antigens. Mezzasoma *et al.* generated protein microarrays by printing microbial antigens to simultaneously determine in human sera antibodies directed against Toxoplasma gondii, rubella virus, cytomegalovirus (CMV), and herpes simplex virus (HSV) types 1 and 2 (ToRCH antigens) (131). Their results showed that the protein microarrays are a suitable assay format for the serodiagnosis of infectious diseases, can be easily optimized for clinical use, and may have potentially important advantages in throughput, convenience and cost.

Several studies have demonstrated that protein arrays can be used for diagnosis of allergies. Hiller *et al.* applied microarray technology to develop a miniaturized allergy test containing 94 purified allergen molecules that represent the most common allergen sources. The allergen microarrays can simultaneously determine and monitor allergic patients' IgE reactivity profiles to large numbers of disease-causing allergens by using single measurements and minute amounts of serum (132). This method may change established practice in allergy diagnosis, prevention, and therapy.

6.9. Cell type classification

Since different cells have distinctive proteins on the cell surface, antibody arrays should be able to distinguish specific expression patterns of different cells. Belov *et al.* developed a rapid, simple procedure, which enables concurrent determination of 50 or more CD antigens on leukocytes or leukemia cells in a single analysis using protein microarrays (133). This technology should allow us to immunophenotype different cell types and will have potential applications in clinical diagnosis.

7. CONCLUSIONS AND PERSPECTIVES

In summary, protein arrays are a powerful tool in biomedical research and hold great promise in clinical applications. Current data already show that protein arrays will have practical applications in diagnosis. Clinical proteomics will become a major backbone in patient care in future clinical practice.

As with any discovery method, the data obtained by protein arrays may not be sufficient to explain the molecular mechanisms of biological processes. However, protein arrays do uncover new molecular targets or new mechanisms in those processes and suggest hypotheses that subsequently can be tested by traditional molecular and cell biological methods or followed with additional scientific exploration.

Protein arrays are possible for many experiments. As protein array technology becomes more stable, easy to perform and inexpensive, an increasing number of experiments will be performed in array format. A caution also must be taken to explain the results. The limitations of protein arrays also must be kept in mind. In the near future and until the technology has been perfected, critical results obtained by this method will still need to be confirmed by more conventional approaches.

Protein array technology is still in its infant state. One of the challenges we are facing in the development of antibody-based protein arrays is the cross-reaction among different antibodies. Therefore, development of other high-specificity and affinity capture reagents that are easy to synthesize and handle is the key to the success of protein arrays. Until we have a library of capture reagents, each of which can specifically bind to a single protein with high specificity, the ability of protein arrays to detect whole sets of proteomes will remain a distant dream.

We will continue to enjoy new technologies and approaches in protein arrays. Three major efforts in protein array technology involve development of capture reagents, development of detection methodology and development of amplification systems.

An inherent problem associated with array technology is the difficulty in comparing experiments from different time periods or from different laboratories. One solution is to develop quantitative protein array technology; then expression levels of different proteins can be measured quantitatively. Such quantitative measurement will allow investigators to compare data from different experiments or from different labs.

Meanwhile, we expect the applications of protein array technology to increase exponentially in the coming years. Other trends will be combination of cDNA microarrays and protein arrays. Due to the ability to simultaneously detect gene expression from whole genomes, we can screen gene expression from cDNA arrays and identify certain genes. This group of genes then can be further tested using protein arrays. Protein arrays can also be combined with protein separation technology (134).

Informatics is the key to analyzing and handling mass data generated from protein arrays. Thanks to the advance of informatics in the DNA array field, most approaches can be directly used or adapted to protein arrays. Analysis tools specially designed for protein arrays will become increasingly important as the popularity of protein arrays increases.

Another future direction of this technology will be to profile *in vivo* gene expression at the single-cell level. A key goal of biology is to relate the expression of specific genes to a particular cell phenotype. Currently, protein arrays for assaying protein expression destroy the structural context. To obtain real time protein expression at the single-cell level, we need to develop an effective way to simultaneously measure multiple protein changes in the same cell. By combining advances in computational fluorescence microscopy with multiplex probe design, Levsky *et al.* (135) developed a technology which can simultaneously visualized multiple gene expression inside single cell. This technology fuses both genomics and cell biology into cellular genomics. Using this technology, they successfully analyzed 11 genes in serum-stimulated cultured cells. Such an approach will greatly influence our understanding of gene expression and cell phenotype.

Lab-in-chip or microfluidics (136-138) technology will continue to be improved. Microfluidic systems are composed of fluid channels and chambers with dimensions of tens to hundreds of micrometers. The unique structure provides a rapid and efficient mixing of components, which is critical in the control of chemical reactions. This technology can be used to monitor the binding events of molecular interactions. Real-time millisecond quantitation of binding kinetics and detection of low-affinity interactions are among the important advantages of this system. Nanofluidic systems, which are three orders of magnitude smaller than microfluidics, have been demonstrated to have the ability to read directly the nucleotide sequence of a single-stranded DNA (139, 140). These technologies will have a great impact on protein arrays.

Research and application in protein array technology is just beginning and the future is bright.

8. ACKNOWLEDGEMENTS

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Abreviations: ELISA: Enzyme linked immunosorbent assay, µCP: micro contact printing, PISA: protein *in situ* array, RCA: rolling circle amplification, SDS PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis,

SELDI: Surface enhanced laser desorption and ionization, SELEX: systematic evolution of ligands by exponential enrichment, SPR: surface plasmon resonance.

Key Words: Protein Arrays, Antibody Arrays, Proteomics, Expression, Protein-Protein Interaction, Protein-DNA Interaction, Antibody, Capture Molecules, Biochip, Modification, Diagnosis, Review

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