Protein Microarray Methods

Recent Developments in Protein Microarray Technology

*David S. Wilson and Steffen Nock**

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The sequencing of the human genome and the advent of DNA chips and sophisticated bioinformatics platforms have enabled molecular biologists to take a more global view of biological systems and to analyze naturally occurring genetic variation. Microarrays of antibodies can measure the concentrations of many proteins quickly and simultaneously. Microarrays of genomically encoded proteins allow scientists to screen entire genomes for proteins that interact with particular factors, catalyze particular reactions, or act as substrates for protein-modifying enzymes or as targets of autoimmune responses. The new protein microarray platforms will prove invaluable to basic biological research, and will dramatically accelerate the pace of discovery of drug targets and diagnostic biomarkers.

Introduction

During the last two decades, protein biochemists have envied molecular biologists working with nucleic acids for their rapid and tangible scientific progress, the most notable example being the sequencing of the human genome. The latest triumph of nucleic acid biotechnology has been the invention of DNA microarrays, which allow for the simultaneous monitoring of the expression, at the mRNA level, of thousands to tens of thousands of genes in a single experiment. These accomplishments have been enabled by significant improvements in the core technologies of nucleic acid biochemistry—oligonucleotide synthesis, DNA sequencing, and hybridization-based detection of DNA and RNA—as well as in bioinformatics. In particular, the ability to miniaturize, multiplex, and automate these basic operations has radically increased the rate of data generation.

Despite the clear technical advantages of analyzing nucleic acids over proteins, the latter are the functional products of most genes and the targets of pharmaceutical intervention, so there is a clear need for protein analysis tools to "catch up" with those for examining nucleic acids. One avenue in this direction has been the development of protein microarrays, which have been reviewed in depth.[1–5] As in the case of nucleic acid microarrays, both multiplexation and miniaturization are achieved relative to traditional methods, thus dramatically increasing the amount of data that can be obtained per volume of biological sample. For example, when 100 μL of sample is applied to a flat surface with 10000 spatially and biochemically distinct features (for example, each one being derivatized with a different antibody—see Figure 1), then it would be possible to obtain up to 10000 data points in one experiment. By comparison, a conventional 96-well ELISA type assay would only produce a single data point from the same amount of sample.

Identification of Molecular Patterns

This ability of protein microarrays to increase the quantity of data points per amount of biological sample is critical for protein expression profiling since the protein samples are often very limited in supply and (unlike nucleic acids) cannot be amplified. If the goal of expression profiling is to identify molecular markers for pathological conditions, protein microarrays will allow for more potential markers to be screened than would be possible using conventional, serial biochemical analysis. This not only offers a higher likelihood of finding a suitable marker, but also opens up the possibility of finding diagnostic patterns ("biosignatures") of protein levels that would be missed if one were taking a more traditional, reductionist approach of attempting to correlate individual protein levels with disease states. The possibility that pathological states can be correlated to patterns of gene expression, as opposed to correlation to single markers, has been clearly demonstrated for various types of cancer at the mRNA level.[6–12] These reports also demonstrated that gene expres-

[*] S. Nock, D. S. Wilson
Department of Biochemistry
Zyomyx, Inc.
26101 Research Road, Hayward, CA 94545 (USA)
Fax: (+1) 510-784-2569
E-mail: snock@zyomyx.com

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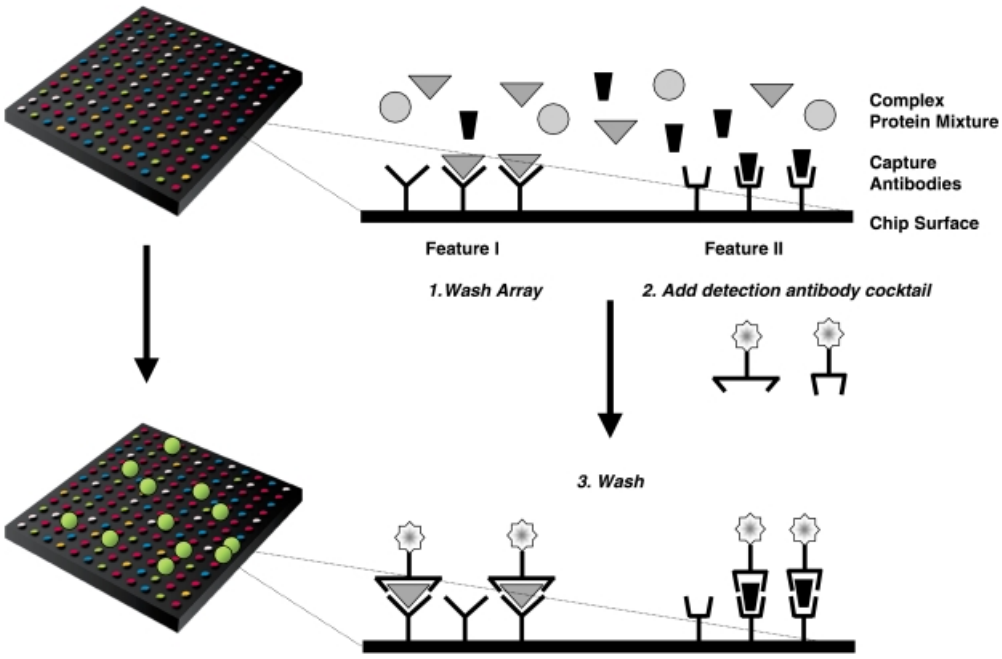

Figure 1. The use of protein microarrays in protein expression profiling. A schematic representation of a 12×14 antibody array is shown top left. Each feature is derivatized by a distinct antibody specifically (top right), making it possible to measure the concentrations of up to 168 proteins simultaneously. First, a complex protein mixture (biological sample) is brought into contact with the antibody array. Each feature on the array captures a single protein species from the complex mixture. After equilibration, the array is washed to remove unbound proteins. At this point, the bound proteins on the array can be directly measured in cases where the analyte proteins have been labeled by fluorescent compounds, for example (this is not explicitly shown). Alternatively, a cocktail of labeled detection antibodies can be added to the array, thus forming sandwich complexes on the features that have captured analyte protein (bottom right). After washing, the signal on the array can be quantified.

sion patterns could taxonomically stratify histologically indistinguishable tumor types, and that these molecular distinctions could correlate to disease prognosis and therapeutic responsiveness.

Patterns of protein levels have also been proven to correlate with pathological states. Petricoin et al.[13] performed surface-enhanced laser desorption and ionization (SELDI) mass spectrometry on hydrophobically enriched serum proteins and peptides from patients with ovarian cancer, and compared the patterns of mass/charge (m/z) signals to those from healthy individuals. The data were analyzed by a novel pattern–recognition method that combines elements from genetic algorithms and cluster analysis to correlate patterns of peak intensities to the pathological condition. The algorithm uncovered a pattern of intensities at five different m/z ratios that was capable of diagnosing malignant ovarian cancer in all diseased patients tested, and had a false positive rate of 5%. This method led to a much higher positive predictive value (94%) than diagnosis based on the most widely used serum biomarker for ovarian cancer, cancer antigen 125 (CA125), which has a positive predictive value of only 10%.

This study demonstrates the benefits of being able to measure concentration differences for numerous proteins in parallel. Mass spectrometry offers one method for making such a comparison, and retains the advantage that patterns

Steffen Nock, born in 1967, studied Biochemistry at the University of Bayreuth (Germany). In 1993 he started his PhD work on guanine nucleotide binding proteins in prokaryotes under the supervision of Prof. M. Sprinzl (Bayreuth), in collaboration with Prof. Roger Goody at the Max-Planck Institut für Molekulare Physiologie in Dortmund. After completion of his doctorate in 1996, he was awarded a fellowship from the Deutsche Forschungsgemeinschaft to carry out research in the Department of Biochemistry at Stanford University. In 1998 he co-founded Zyomyx, Inc., a biotechnology company that develops protein microarrays.

David S. Wilson was born in 1965 in New York, and earned his B.S. in Biochemistry from the University of California, Berkeley. He received his Ph.D. from the Rockefeller University in Biochemistry and Biophysics under the supervision of Claude Desplan and John Kuriyan. In 1996, he received a post-doctoral fellowship from the Cancer Research Foundation of the Damon Runyon-Walter Winchell Foundation in the laboratory of Jack W. Szostak at the Massachusetts General Hospital/Harvard Medical School. He moved to Zyomyx, Inc., in 2000, and is currently the Manager of Protein Engineering.

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can be obtained without knowledge of the proteins that correspond to the *m*/*z* signals. Disadvantages of this method include low throughput relative to immunoassays, and difficulty in molecular identification of the markers. When markers can be identified, however, antibodies could be developed and incorporated into multiplexed immunoassays, which are compatible with much higher throughput and offer greater sensitivity and accurate quantification. A powerful synergy therefore exists between discovery technologies such as mass spectrometry and high-throughput, quantitative immunoassays for testing thousands of samples from different patient populations.

Miniturized Immunoassays

The ability to miniaturize and multiplex immunoassays for measuring protein abundance has been the subject of numerous studies and reviews.[3–5] Recently, Schweitzer et al.[14] demonstrated a microarray system with antibodies that can be used for the simultaneous measurement of 75 cytokines (soluble signaling proteins involved in immune response). Antibodies of known specificities were arrayed onto a glass slide, which was subsequently incubated with biological samples, washed, and then exposed to a cocktail of detection antibodies (Figure 1). The detection antibodies were also of known specificity, and could bind to different regions (epitopes) of the cytokines than those bound by the surface-immobilized antibodies. Therefore, cytokines in the biological sample could be "sandwiched" between the surface-attached and detection antibodies, thus causing retention of the detection antibodies after washing. Detection antibodies are typically labeled with fluorescent dyes, which allows for measurement by confocal fluorescent scanners designed for reading DNA microarrays. The sensitivity of this method, unfortunately, may be insufficient in some cases to measure proteins present at very low concentrations such as cytokines.

To address this limitation, a powerful signal-enhancement method called rolling circles amplification (RCA) was used. The mechanism of this clever method is beyond the scope of this review, but consists of using a sandwich immunoassay in which the detection antibodies are conjugated to DNA sequences. Once the detection antibodies are localized to the antigens on the protein array features, their DNA sequences are extended by a DNA polymerase in situ, thus forming long DNA polymers of defined sequence that are tethered to the detection antibody and therefore to the appropriate feature of the microarray. After this polymerization step, the extended DNA sequence is allowed to hybridize to fluorescently labeled DNA oligonucleotides of complementary sequence. Since the extended DNA polymers are very long, multiple oligonucleotides, and therefore fluorophores, are attached to each detection antibody.

By using this RCA–sandwich approach, Schweitzer et al.[14] were able to detect cytokine concentrations in the pico- and subpicomolar ranges, which should be sufficient to measure biological levels of these proteins. This technology was used to monitor the expression levels of 51 cytokines in cultured dendritic cells in response to stimulation by either lipopolysaccharide or tumor necrosis factor α. Cytokine concentrations were measured on only a few microliters of cell culture supernatant at multiple time points, a project that would have required thousands of times more sample using standard ELISA methods. The authors observed the upregulation of several cytokines, and validated the data by comparison to standard ELISA methods, which showed comparable sensitivity.

As an alternative to using methods based on nucleic acid amplification to obtain sufficient assay sensitivity to measure biologically relevant analyte concentrations, we have obtained comparable sensitivity by using an extremely bright fluorescent label (phycoerythrin) for the detection of antibodies and by optimizing the surface chemistry and antibody engineering (Figure 2). In this way, it is possible to create a protein-resistant surface with a densely packed monolayer of oriented antibody fragments that are nearly 100% active.[15]

Commercial antibody arrays with hundreds of distinct antibodies are now available from BD Biosciences Clontech (Palo Alto, CA) and Hypromatrix (Millbury, MA). To use these products, one must label the proteins in the biological sample (with a fluorophore, for example), expose them to the array, wash, and then detect the labeled proteins that bind to the corresponding antibody features. Alternatively, one can compare two different samples (such as normal versus pathological) by labeling the proteins from each sample with a different fluorophore, mixing the samples, and then applying them to the arrays[16] (Figure 3). In this case, one compares the relative fluorescence intensity at each antibody feature so as to identify differences in protein levels between the two samples. Two drawbacks of this type of approach are that sensitivity is generally not very high, which makes it difficult to detect proteins present at low concentration, and sample-labeling efficiency is often variable.[16]

Antibody–antigen interactions can also be used to study immune responses by applying patient serum to microarrays of immobilized antigens (Figure 4), thus providing information on the patient's exposure to pathogens, allergic reactions, or the presence of autoimmune disorders. Robinson et al.[17] created a microarray of 196 known autoantigens and tested serum from patients with 6 different autoimmune disorders, and found a pattern of reactivity that could differentiate the syndromes from each other and from normal individuals. Furthermore, the results matched those obtained by conventional ELISA methods. The isotype subclass of the autoantibodies could also be determined by developing the arrays by using different subclass-specific antibodies with distinct fluorophores. For most of the autoantigens it was possible to detect physiological concentrations of autoantibodies, with lower limits of detection than could be obtained by conventional ELISA methods. Some of the antigens were unrecognizable, however, presumably because of denaturation resulting from the immobilization method. The authors suggest that this problem could be overcome by replacing the surface (polylysine-coated glass, which binds proteins nonspecifically) with one that is more biocompatible. Nitrocellulose-coated glass slides have been successfully used to array carbohydrate-based antigens and monitor human

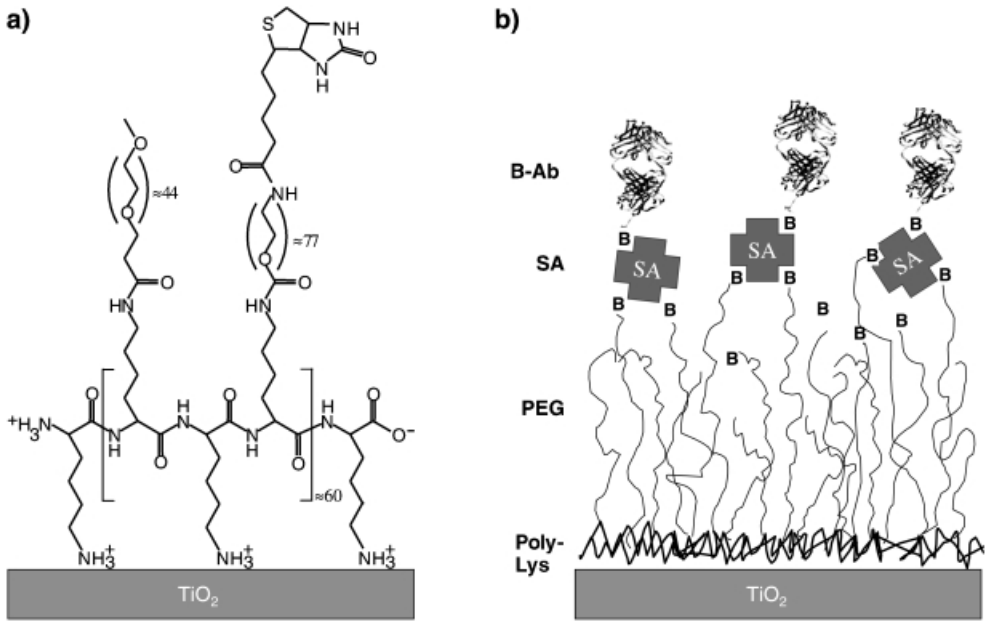

Figure 2. A surface chemistry that is highly resistant to nonspecific protein binding, but allows for derivitization with antibodies. a) Structure of a poly(L-lysine-PEG-biotin) copolymer adsorbed onto a negatively charged surface such as TiO_2. The methoxy-PEG and biotin-PEG chains are grafted onto about 20 and 9% of the lysine side chains, respectively. b) Schematic representation of this surface after derivitization with streptavidin (SA). Since SA is tetravalent, biotinylated antibodies (B-Ab) or Fab fragments (as shown) can be attached through the free biotin-binding sites of SA.

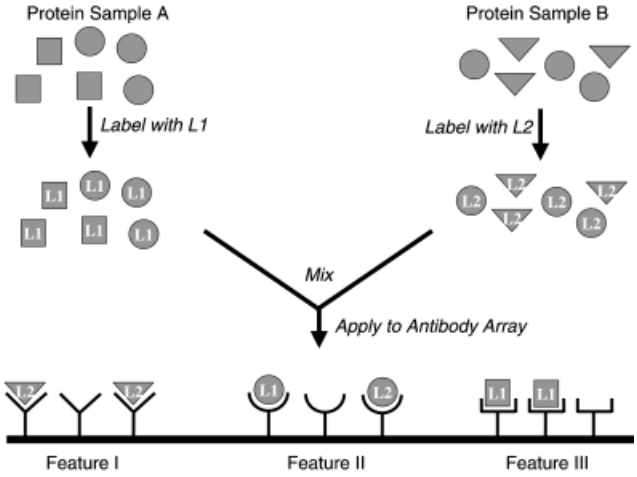

Figure 3. Differential protein expression profiling. Two different protein samples (normal and pathological) are labeled with two different fluorescent dyes, L1 and L2. The two samples are then mixed and applied to an antibody array, which allows the labeled proteins from each sample to be captured by antibodies. Feature I will display a high signal for L2 but not L1, which indicates that protein sample B has a higher concentration of the corresponding protein than does sample A. The opposite is true for Feature III. Feature II shows equal activity of L1 and L2, which indicates there are equal concentrations of the corresponding proteins in the samples.

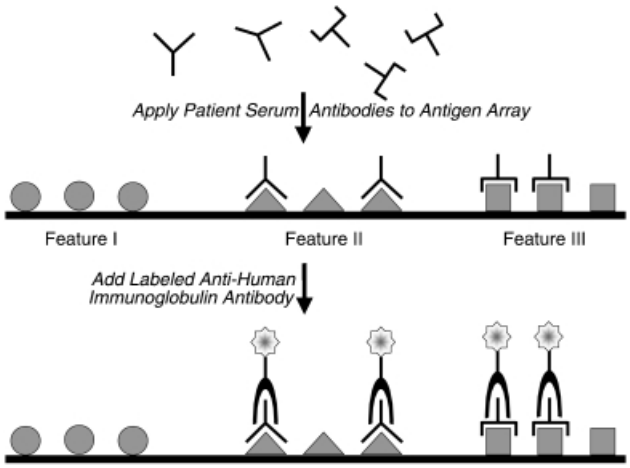

Figure 4. Probing immune responses with antigen arrays. These arrays are inverted with respect to those shown in the previous figures. Patient serum, which contains immunoglobulins (antibodies), is exposed to an antigen array so that antibodies specific for the different antigens are captured at specific locations on the array. After washing away unbound immunoglobulins, a labeled anti-human immunoglobulin antibody is added, and binds to the human antibodies on the array, thus indirectly revealing the antibody response to the various antigens in the patient serum.

immune responses.[18] This result is important since polysaccharides frequently constitute the most immunogenic components of infectious agents.

Other possible applications of antigen arrays could include discovery of cancer-specific autoantigens, which could lead to novel diagnostic methods and to drug target candidates. Antigen arrays could also enable a more systematic approach to vaccine development.

New Microarray Formats

In addition to measuring the abundance of proteins and antibodies, arrays can also be used to study protein activity. Zhu et al.[19] created expression constructs for virtually all of the 5800 genes in yeast, and then expressed and purified small quantities of them in parallel, and finally arrayed them onto glass slides. These "proteome chips" were subsequently

incubated with labeled molecules to identify yeast proteins with which they interact. As a result, they identified several proteins that bind to the protein calmodulin and to certain lipids.

Enzymatic activity can also be studied in microarray format. Arrays of peptide substrates and kinases were used to characterize the specificity of virtually all of the known yeast protein kinases, for example.[20] Recently, Houseman et al.[21] described a system for studying enzyme–substrate interactions on a type of surface that provides the chemical characteristics consistent with quantitative assays: it is protein-repellent and has a defined density and homogeneous presentation of covalently immobilized substrates. This was achieved by self-assembly of organic thin films composed of alkanethiols with polyethyleneglycol (PEG) groups, which are known to be resistant to nonspecific protein binding, onto gold surfaces. A fraction of the PEG groups on this surface contain an ω-benzoquinone functionality that can form a covalent bond with cyclopentadiene-conjugated peptides. The peptide substrates are thereby immobilized on a well-characterized and homogeneous surface, thus minimizing the influence of the surface on the enzyme–substrate interactions. The authors showed that the peptides could be enzymatically phosphorylated by a protein kinase, thus providing information about substrate specificity and phosphorylation kinetics. Phosphorylation was measured either directly, by incubating the immobilized peptides with the kinase and radiolabeled [γ-^{32}P]ATP (ATP = adenosine 5′-triphosphate), or indirectly, by using a fluorescently labeled antibody against phosphotyrosine. This system was also used to quantify inhibitory constants of different kinase inhibitors.

One of the greatest challenges in the protein microarray field will be the incorporation of membrane proteins, which collectively represent about half of the current drug targets.[22] A method for immobilizing membrane proteins onto microarrays has recently been described. Fang et al.[22] showed that aminopropylsilane-derivatized glass slides could be used to immobilize lipid bilayers in such a way that they were stable against washing in aqueous buffers and against dehydration, but retained the capacity to support long-range lateral fluidity. The authors demonstrated that G-protein-coupled receptors, which constitute one of the most important classes of drug targets, could be immobilized on the glass slides and retain their ligand-binding affinity and specificity.

Ion channels are one of the most important classes of membrane proteins, and microarray formats for measuring current through such channels are being developed. The membranes must be immobilized in such a way as to maintain their insulation properties so that conductance of ions is largely the result of the embedded channel proteins rather than of defects in the bilayer structure.[23] Arrays of different channel proteins embedded in such supported membranes, in which each feature could be independently monitored by a microelectrode, would enable the screening of compounds for their ability to modulate the conductive properties of numerous channel proteins in parallel.

Another new concept in the protein microarray field is referred to as "affinity contact printing."[24] The basis for this technology is to use a three-dimensional chip in which raised, flat structures (mesas) protrude from the base of the chip (Figure 5). Different proteins can then be covalently attached to the different raised features. The chip is then brought into contact with a complex protein solution so that the covalently attached proteins on the tops of the mesas can capture proteins from the complex mixture. The chip thereby acts as a purifying and concentrating device. Next, the mesa chip is removed from the protein solution, washed, and then used as a stamp: it is brought into contact with a surface that has a high, nonspecific affinity for proteins. The specifically bound proteins are thereby transferred to the new surface (the "printed surface"), while the covalently attached proteins are left on the stamp. The printed surface can then be analyzed by mass spectrometry or other methods. Some of the printed proteins retain their biological activity.

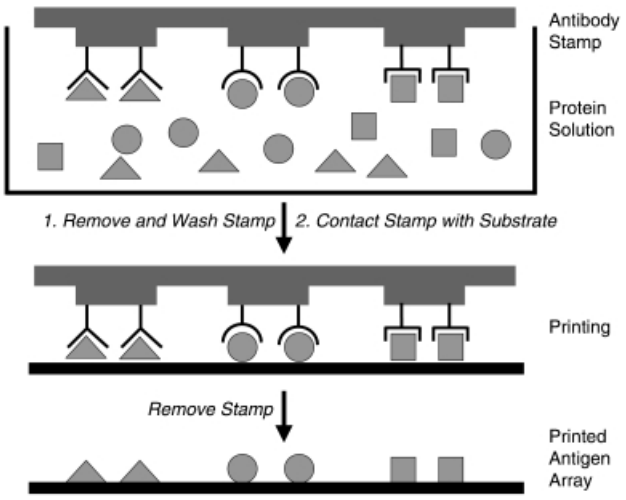

Figure 5. Affinity contact printing. A stamp is prepared whereby antibodies (or other affinity agents) are covalently attached to protruding structures. This stamp is then "inked" by being brought into contact with a protein solution. After washing, the inked stamp is brought into contact with a substrate that binds to proteins with a higher affinity than that of antibody–antigen interactions, which results in the printing of specific proteins in defined patterns onto a substrate.

Use of Protein Microarrays in Drug Development

The advanced target discovery technologies of genomics and proteomics have led to a vast amount of potential drug targets. Nearly all of these molecules are proteins. This situation has created an immediate need for tools that help to validate and prioritize these potential targets. New tools also need to address other bottlenecks in drug development—the preclinical and clinical stages, which have to be streamlined and made more efficient.

Protein microarrays hold great promise for accelerating the development of both therapeutic and diagnostic biomarkers. Figure 6 outlines the different stages of these processes and highlights the areas where protein biochips could be most relevant.

Protein microarrays currently have minor impact on the initial phase of target discovery, since there are a limited

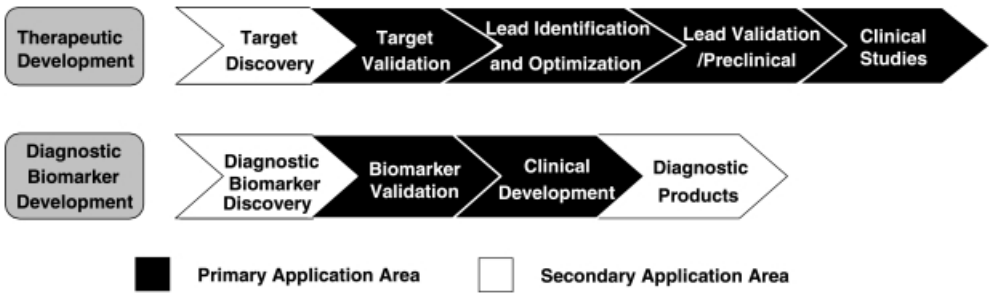

Figure 6. The use of protein microarrays for the development of drug and diagnostic biomarkers.

number of antibodies available to profile potential targets. Rather, genomics and proteomics technologies, such as DNA arrays for mRNA expression profiling, and two-dimensional gel electrophoresis in combination with mass spectrometry, are uncovering numerous drug target candidates. Once these targets have been defined, they must be placed into a biological context, for example, into certain pathways. A combination of mass spectrometry and protein chips with immobilized targets that can act as bait proteins to capture protein complexes will help to streamline and multiplex the placement of these proteins into pathways, and will also further define pathways and cross-talk between them. Once the biological context of a target has been defined, a statistical validation across different tissues of many patients has to be performed. This is an area where protein expression profiling chips based on simple, quantitative readout (such as fluorescence) could take over from the lower throughput, less quantitative techniques based on two-dimensional gels and mass spectrometry.

Protein microarrays will also help to speed up lead identification as well as downstream processes such as lead optimization and validation. The inhibitory properties of lead compounds on enzymatic drug targets, and also on related nontarget enzymes, can be analyzed in chip-based systems.[21,25,26] Substantial savings could be made both in terms of time and precious reagents by multiplexing and miniaturizing these assays. Generic chips to assess toxic side effects of drug candidates early on in the development process will potentially help to prevent failures during the ever more costly later stage clinical studies.

Biomarker discovery, which is tightly linked to the drug development process, constitutes a second area where protein arrays could dramatically accelerate the pace of research. Biomarkers can support preclinical and clinical development, help identify focused subgroups in the clinical patient population with predispositions relating to efficacy or toxicity, and be used to monitor efficacy and toxicity of drug compounds. Examples of the utility of protein chips as tools to discover biomarkers in autoimmunity[17] and cancer[13] were discussed above. We and other research groups are developing antibody arrays to measure cytokines and other mediators of the immune response with the hope of finding patterns that will aid in the diagnosis and treatment of inflammatory diseases.

In conclusion, protein microarrays have the potential to revolutionize many stages of drug and biomarker development. This will lead to significant savings in cost, time, and reagents. Furthermore the use of protein biochips will enable pharmaceutical companies to personalize therapeutic regimens to the appropriate patient groups.

Summary and Outlook

Protein microarrays have gained popularity over the last two to three years, with dozens of reports in the literature of their use (reviewed in references [1–5]). Several basic platforms have been established, especially for expression profiling applications. The most significant challenge for the future will be to obtain the biological content with which to load the chips.[2,27] Expression profiling requires at least one specific antibody for each protein that is to be monitored. Polyclonal antibodies may not be specific enough for use in multiplexed immunoassays, but the development of monoclonal antibodies requires a great deal of time (usually about six months) and labor. One attractive alternative is to use phage display of recombinant antibody fragments, an artificial in vitro selection process by which high affinity binding agents can be obtained from large, naïve libraries.[28–31] The time required to perform binding selections is much shorter than that to generate a robust immune response in an animal, and there is no problem with toxic or self proteins.

Other artificial selection methods include microbial display[32] and ribosome display[33] of antibody fragments, mRNA display of artificial protein libraries,[34] the two-hybrid systems,[35] and systematic evolution of ligands by exponential enrichment (SELEX) of nucleic acid libraries.[36,37] These are all relatively new systems with the potential to be automated.

Before one can generate a binding agent by any of these methods, the target protein must be synthesized and purified. Most mammalian proteins can be expressed at high levels in *E. coli*, or in vitro using *E. coli* extracts, but they usually precipitate as insoluble, misfolded proteins. For most human proteins, it will therefore be necessary to access alternative expression systems or identify robust, high-throughput protein-refolding protocols for bacterially expressed proteins.

An easier route to antigen generation would be to use short synthetic peptides, but antibodies for short peptides are generally lower in affinity than are those that are raised against folded proteins. The development of detection methods applicable to protein arrays with large numbers (hundreds to thousands) of different antibody specificities is

another area in need of innovation. One can chemically label the complex mixtures to be analyzed with fluorescent compounds, but this may provide insufficient sensitivity for proteins present at low concentrations because of a poor signal-to-noise ratio. Many important signaling molecules (such as cytokines) can be present in human serum at about a billionfold lower concentrations than other proteins in the same mixture, and so even high-quality immobilized antibodies will generally not be capable of distinguishing the specific signal from the general noise arising from nonspecific protein binding. The signal-to-noise ratio can be dramatically improved by utilizing a sandwich assay, but this requires the development of two binding reagents per target protein.

The last few years have witnessed intense innovation in the field of protein microarrays, but substantially more development will be required before this technology reaches its zenith in contributing to both basic biological research and drug development.

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[1] R. E. Jenkins, S. R. Pennington, *Electrophoresis* **2001**, *1*, 13 – 29.
[2] T. Kodadek, *Chem. Biol.* **2001**, *8*, 105 – 115.
[3] D. J. Cahill, *J. Immunol. Methods* **2001**, *250*, 81 – 91.
[4] D. S. Wilson, S. Nock, *Curr. Opin. Chem. Biol.* **2001**, *6*, 81 – 85.
[5] D. Stoll, M. F. Templin, M. Schrenk, P. C. Traub, C. F. Vohringer, T. O. Joos, *Front. Biosci.* **2002**, *7*, c13-c32.
[6] T. R. Golub, D. K. Slonim, P. Tamayo, C. Huard, M. Gaasenbeek, J. P. Mesirov, H. Coller, M. L. Loh, J. R. Downing, M. A. Caligiuri, C. D. Bloomfield, E. S. Lander, *Science* **1999**, *286*, 531 – 537.
[7] A. A. Alizadeh, M. B. Eisen, R. E. Davis, C. Ma, I. S. Lossos, A. Rosenwald, J. C. Boldrick, H. Sabet, T. Tran, X. Yu, J. I. Powell, L. Yang, G. E. Marti, T. Moore, J. Hudson, Jr., L. Lu, D. B. Lewis, R. Tibshirani, G. Sherlock, W. C. Chan, T. C. Greiner, D. D. Weisenburger, J. O. Armitage, R. Warnke, L. M. Staudt, *Nature* **2000**, *403*, 503 – 511.
[8] C. M. Perou, T. Sorlie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, C. A. Rees, J. R. Pollack, D. T. Ross, H. Johnsen, L. A. Akslen, O. Fluge, A. Pergamenschikov, C. Williams, S. X. Zhu, P. E. Lonning, A. L. Borresen-Dale, P. O. Brown, D. Botstein, *Nature* **2000**, *406*, 747 – 752.
[9] T. Sorlie, C. M. Perou, R. Tibshirani, T. Aas, S. Geisler, H. Johnsen, T. Hastie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, T. Thorsen, H. Quist, J. C. Matese, P. O. Brown, D. Botstein, L. P. Eystein, A. L. Borresen-Dale, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 10869 – 10874.
[10] S. M. Dhanasekaran, T. R. Barrette, D. Ghosh, R. Shah, S. Varambally, K. Kurachi, K. J. Pienta, M. A. Rubin, A. M. Chinnaiyan, *Nature* **2001**, *412*, 822 – 826.
[11] T. O. Nielsen, R. B. West, S. C. Linn, O. Alter, M. A. Knowling, J. X. O'Connell, S. Zhu, M. Fero, G. Sherlock, J. R. Pollack, P. O. Brown, D. Botstein, M. van de Rijn, *Lancet* **2002**, *359*, 1301 – 1307.
[12] S. L. Pomeroy, P. Tamayo, M. Gaasenbeek, L. M. Sturla, M. Angelo, M. E. McLaughlin, J. Y. Kim, L. C. Goumnerova, P. M. Black, C. Lau, J. C. Allen, D. Zagzag, J. M. Olson, T. Curran, C. Wetmore, J. A. Biegel, T. Poggio, S. Mukherjee, R. Rifkin, A. Califano, G. Stolovitzky, D. N. Louis, J. P. Mesirov, E. S. Lander, T. R. Golub, *Nature* **2002**, *415*, 436 – 442.
[13] E. F. Petricoin, A. M. Ardekani, B. A. Hitt, P. J. Levine, V. A. Fusaro, S. M. Steinberg, G. B. Mills, C. Simone, D. A. Fishman, E. C. Kohn, L. A. Liotta, *Lancet* **2002**, *359*, 572 – 577.
[14] B. Schweitzer, S. Roberts, B. Grimwade, W. Shao, M. Wang, Q. Fu, Q. Shu, I. Laroche, Z. Zhou, V. T. Tchernev, J. Christiansen, M. Velleca, S. F. Kingsmore, *Nat. Biotechnol.* **2002**, *20*, 359 – 365.
[15] a) L. A. Ruiz-Taylor, T. L. Martin, F. G. Zaugg, K. Witte, P. Indermuhle, S. Nock, P. Wagner, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 852 – 857; b) P. Peluso, D. S. Wilson, D. Do, H. Tran, M. Venkatasubbaiah, D. Quincy, B. Heidecker, K. Poindexter, N. Tolani, M. Phelan, K. Witte, P. Wagner, S. Nock, *Anal. Biochem.* **2003**, *312*, 113 – 124
[16] B. B. Haab, M. J. Dunham, P. O. Brown, *Genome Biol.* **2001**, *2*, research 0004.1 – 0004.13.
[17] W. H. Robinson, C. DiGennaro, W. Hueber, B. B. Haab, M. Kamachi, E. J. Dean, S. Fournel, D. Fong, M. C. Genovese, H. E. de Vegvar, K. Skriner, D. L. Hirschberg, R. I. Morris, S. Muller, G. J. Pruijn, W. J. van Venrooij, J. S. Smolen, P. O. Brown, L. Steinman, P. J. Utz, *Nat. Med.* **2002**, *8*, 295 – 301.
[18] D. Wang, S. Liu, B. J. Trummer, C. Deng, A. Wang, *Nat. Biotechnol.* **2002**, *20*, 275 – 281.
[19] H. Zhu, M. Bilgin, R. Bangham, D. Hall, A. Casamayor, P. Bertone, N. Lan, R. Jansen, S. Bidlingmaier, T. Houfek, T. Mitchell, P. Miller, R. A. Dean, M. Gerstein, M. Snyder, *Science* **2001**, *293*, 2101 – 2105.
[20] H. Zhu, J. F. Klemic, S. Chang, P. Bertone, A. Casamayor, K. G. Klemic, D. Smith, M. Gerstein, M. A. Reed, M. Snyder, *Nat. Genet.* **2000**, *26*, 283 – 289.
[21] B. T. Houseman, J. H. Huh, S. J. Kron, M. Mrksich, *Nat. Biotechnol.* **2002**, *20*, 270 – 274.
[22] Y. Fang, A. G. Frutos, J. Lahiri, *J. Am. Chem. Soc.* **2002**, *124*, 2394 – 2395.
[23] G. Wiegand, N. Arribas-Layton, H. Hillebrandt, E. Sackmann, P. Wagner, *J. Phys. Chem. B* **2002**, *106*, 4245 – 4254.
[24] A. Bernard, D. Fitzli, P. Sonderegger, E. Delamarche, B. Michel, H. R. Bosshard, H. Biebuyck, *Nat. Biotechnol.* **2001**, *19*, 866 – 869.
[25] C. B. Cohen, E. Chin-Dixon, S. Jeong, T. T. Nikiforov, *Anal. Biochem.* **1999**, *273*, 89 – 97.
[26] P. Arenkov, A. Kukhtin, A. Gemmell, S. Voloshchuk, V. Chupeeva, A. Mirzabekov, *Anal. Biochem.* **2000**, *278*, 123 – 131.
[27] A. Abbott, *Nature* **2002**, *415*, 112 – 114.
[28] T. J. Vaughan, A. J. Williams, K. Pritchard, J. K. Osbourn, A. R. Pope, J. C. Earnshaw, J. McCafferty, R. A. Hodits, J. Wilton, K. S. Johnson, *Nat. Biotechnol.* **1996**, *14*, 309 – 314.
[29] M. D. Sheets, P. Amersdorfer, R. Finnern, P. Sargent, E. Lindquist, R. Schier, G. Hemingsen, C. Wong, J. C. Gerhart, J. D. Marks, E. Lindqvist, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 6157 – 6162.
[30] H. J. de Haard, N. van Neer, A. Reurs, S. E. Hufton, R. C. Roovers, P. Henderikx, A. P. de Bruïne, J. W. Arends, H. R. Hoogenboom, *J. Biol. Chem.* **1999**, *274*, 18218 – 18230.
[31] A. Knappik, L. Ge, A. Honegger, P. Pack, M. Fischer, G. Wellnhofer, A. Hoess, J. Wolle, A. Plückthun, B. Virnekäs, *J. Mol. Biol.* **2000**, *296*, 57 – 86.
[32] K. D. Wittrup, *Curr. Opin. Biotechnol.* **2001**, *12*, 395 – 399.
[33] J. Hanes, C. Schaffitzel, A. Knappik, A. Plückthun, *Nat. Biotechnol.* **2000**, *18*, 1287 – 1292.
[34] D. S. Wilson, A. D. Keefe, J. W. Szostak, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 3750 – 3755.
[35] P. Colas, B. Cohen, T. Jessen, I. Grishina, J. McCoy, R. Brent, *Nature* **1996**, *380*, 548 – 550.
[36] E. N. Brody, L. Gold, *J. Biotechnol.* **2000**, *74*, 5 – 13.
[37] M. P. Robertson, A. D. Ellington, *Nat. Biotechnol.* **2001**, *19*, 650 – 655.