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Affiliations

[†]Author for correspondence
Invitrogen Corporation,
ProtoArray Research
& Development & Operations,
ProtoArray Center, 688 East Main
Street, Branford, CT 06405, USA
Tel.: +1 203 848 1123
barry.schweitzer@invitrogen.com

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Biomarker discovery using protein microarray technology platforms: antibody-antigen complex profiling

Dawn Mattoon, Gregory Michaud, Janie Merkel and Barry Schweitzer[†]

Protein microarrays represent an important new tool in proteomic systems biology. This review focuses on the contributions of protein microarrays to the discovery of novel disease biomarkers through antibody-based assays. Of particular interest is the use of protein microarrays for immune response profiling, through which a disease-specific antibody repertoire may be defined. The antigens and antibodies revealed by these studies are useful for clinical assay development, with enormous potential to aid in diagnosis, prognosis, disease staging and treatment selection. The discovery and characterization of novel biomarkers specifically tailored to disease type and stage are expected to enable personalized medicine by facilitating preventative medicine, predictive diagnostics and individualized curative therapies.

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The molecular era of scientific discovery has championed the philosophy that the incredible diversity of biology has discrete quantifiable limits, functionally categorized as the genome, transcriptome and proteome. The challenge of systems biology is to integrate the wealth of molecular information, model physiologic behavior and create practical methods for improving human health. The Human Genome Project was the first to aim for complete characterization of one of these discrete biologic segments. Initiated in 1990, this ambitious 13-year endeavor established a consortium of research centers with the goal of determining gene sequence information for all of the 20,000–25,000 human genes [1]. Successful completion of the project in 2003 has increased our understanding of the make-up of the genome, and has empowered researchers with tools for developing new assays to further interrogate biologic processes. A tangible outcome of the genome project was the emergence of DNA arrays for the comprehensive evaluation of cellular transcription levels in complex samples. The utility and availability of microarrays for gene expression profiling has resulted in a wealth of information on the transcriptional profile for a variety of disease

states. With efforts to integrate globally generated transcriptional profiling information into publicly available databases, greater consensus has been reached over the number of genes that are actually transcribed. The fact that mRNA abundance is not highly predictive of protein levels in cells suggests that DNA microarray technology for profiling gene expression has limited value in systems biology [2]. Proteomics hopes to fill this gap through the development and application of technologies that provide a comprehensive picture of all human proteins.

In contrast to the information storage and transmission roles of nucleic acids, the proteome provides a direct window through which to view active biomolecules. As such, the proteome can be interrogated for functional relationships that play a role in the maintenance of human health and the development and progression of human disease. Protein-based biomarkers for disease may prove to be of great clinical importance due to their potential in aiding diagnosis, prognosis and assessment of therapeutic options, as well as in tracking disease progression and treatment efficacy. To date, mass spectrometric identification of proteins, separated by either

2D electrophoresis or by liquid chromatography (LC), has been the preferred method for profiling protein abundance. However, the large amount of sample that is required by mass spectrometry (MS), and the limited sensitivity of this approach, makes it almost impossible to routinely investigate proteins that are present at very low levels in blood. Indeed, rare proteins, including classic cancer biomarkers for breast, prostate or ovarian carcinoma, are routinely missed in published studies [3]. Additionally, the time and expertise required for analysis of proteins of interest by MS, combined with the cloning and expression required for validation studies, are bottlenecks that limit the high-throughput potential of this approach. Recently, improvements in protein microarray technology, through the development of arrays containing hundreds to thousands of proteins, simplified protocols and highly sensitive assays have made this platform very attractive for biomarker discovery. Protein microarray content comes in many forms, including microscale samples of tissue, cells, cellular lysates or purified antigens arrayed in discrete locations on a solid support. The discovery of protein-based biomarkers represents a central aim of proteomic systems biology, and is a critical component for enabling personalized medicine. This review will focus on recent advances in the development of affinity profiling utilizing the protein microarray platform for the purpose of biomarker discovery and characterization.

Affinity profiling

Antibodies comprise an important class of protein affinity and detection reagents, with tremendous utility in a range of biologic assays, including flow cytometry, immunoblotting, immunohistochemistry, immunocytochemistry and enzyme-linked immunosorbent assays (ELISA). Purified antibodies have been used to identify proteins within simple and complex samples.

Conversely, proteins can be used to identify antibodies present in simple solutions or in more complex samples, such as blood. Disease-specific proteins and antibodies identified through proteomic affinity profiling have tremendous potential as biomarkers due to their expected ability to reduce the time and costs of clinical trials, and to help provide a molecular rationale of disease type, stage and progression.

Humoral antibody production is the immune response mounted against invading pathogens, environmental allergens or self antigens. In a simplified view, self proteins or nonself antigens such as allergens or proteins produced by invading microorganisms, such as bacteria and viruses, represent disease biomarkers. Additionally, the presence, levels and activities of antibodies directed against these proteins can be exploited as biomarkers. As such, several diagnostic immunoassays that detect antibodies resulting from infection or exposure have been developed and are widely employed in clinical settings [4].

Antibody-mediated immunity directed against proteins involved in malignant transformation has been described for nearly all human tumor types [5]. Apoptotic tumor cells release proteins that are picked up and displayed by antigen-presenting cells (APCs). T-helper cells interact specifically with APCs and are stimulated to release cytokines, which induce T-cell proliferation and stimulate B-cells, which are localized at the site of the tumor, through antigen-specific binding. These activated B-cells multiply and differentiate, resulting in the production of antitumor autoantibodies (FIGURE 1). These autoantibodies can be disease-specific indicators if the antigens recognized are only expressed in that particular disease type. Alternatively, these autoantibodies can be a component of the immune response against a self antigen that is expressed in a variety of cancer types. For example, serologic responses against the cancer/testis antigen NY-ESO-1 have been observed in multiple

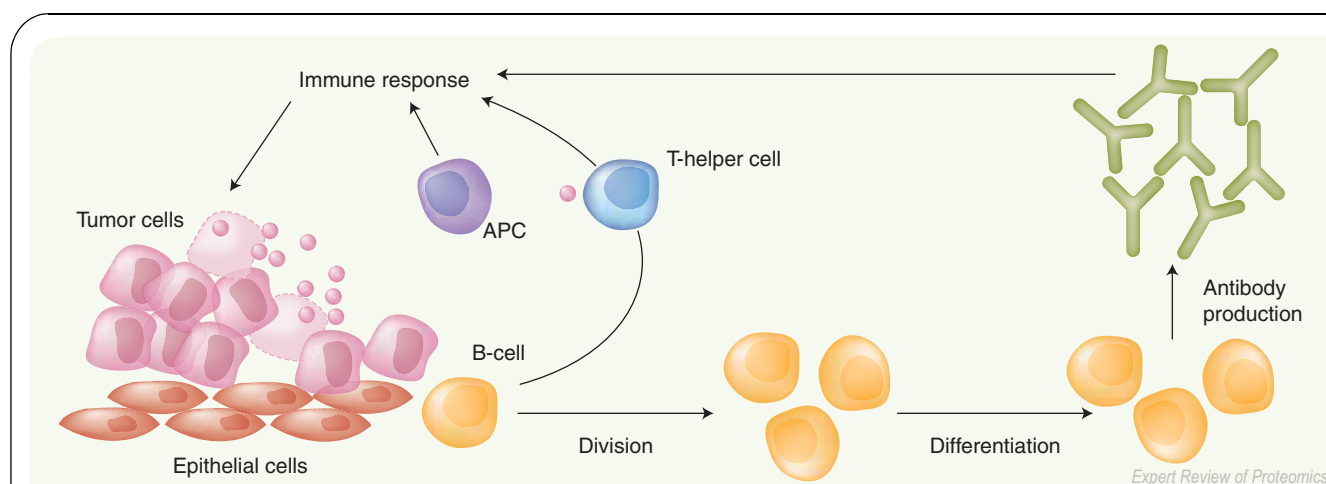


Figure 1. Humoral response to cancer. Proteins expressed by tumor cells are processed by APCs, which activate T-helper cells through a specific binding event. T-helper cells, in turn, activate B-cells localized to the tumor site through antigen-specific binding. Cytokines released by activated T-helper cells stimulate B-cells to divide and differentiate into plasma cells. These differentiated B-cells produce antibodies directed against tumor antigens, which can be detected through immune response profiling on protein microarrays. Adapted with permission from Invitrogen Corporation © 2005.

APC: Antigen-presenting cell.

cancer types, and have been demonstrated to correlate with poor prognosis [6,7]. An idealized representation of an autoantibody response to cancer is shown in FIGURE 2. Antibodies directed against tumor proteins arise early in the tumor development process, and are potential biomarkers for early disease detection. Antitumor antibody titers rise during disease progression and, in the idealized model, fall following treatments that result in tumor cell death. Patients could be monitored for the presence and titer of antitumor antibodies in order to facilitate early detection of disease and to aid in disease prognosis. The sensitivity of detection makes autoantibody profiling a particularly attractive platform for identifying diagnostic biomarkers. However, changes in protein expression patterns in recurrent disease suggests the autoantibody profile may also evolve during disease progression. Identification of stage-specific autoantibody profiles is an important challenge for long-term disease monitoring using protein microarray technology.

An autoimmune response that results in the inappropriate activation of the immune system leads to the generation of antibodies directed against self proteins. Several diseases have been characterized by the presence of autoantibodies, such as lupus and rheumatoid arthritis. These autoimmune disorders are highly complex and are usually the consequence of an intricate interplay between environmental factors and multiple gene products within a patient's specific genetic context [8]. Studies have demonstrated the presence of autoantibodies in systemic lupus erythematosus patients prior to the onset of clinical disease. These findings highlight the potential for an autoimmune signature to be utilized in diagnostic assays designed for early disease detection [9].

In summary, biomarkers may include external proteins derived from allergens or invading pathogens, tumor-derived proteins, human autoantigens or the antibodies raised against these proteins. These molecules can be assayed using non-invasive sampling techniques, and evaluated in simple blood tests. Since immune response profiling specifically identifies immunogenic proteins, rather than proteins that simply exhibit elevated levels in the disease population, such as those identified through mass spectroscopic analysis, biomarkers identified through immune response profiling are more likely to represent good candidates for vaccine development. Microarray technology, which requires small amounts of sample and is amenable to high throughput, is well suited for evaluating antibody immune response profiles for the discovery of novel disease biomarkers.

Microarray technology for biomarker discovery

Whole-cell microarrays

Surgical pathology has long made use of paraffin-embedded tumor biopsies for immunohistochemical analysis. This concept has recently been adapted to the microarray format and standardized for high-throughput manufacture [10,11]. Affinity profiling of tissue microarrays (TMAs) has been successfully employed to identify multiple biomarkers for a variety of diseases, including prostate cancer, breast cancer and melanoma,

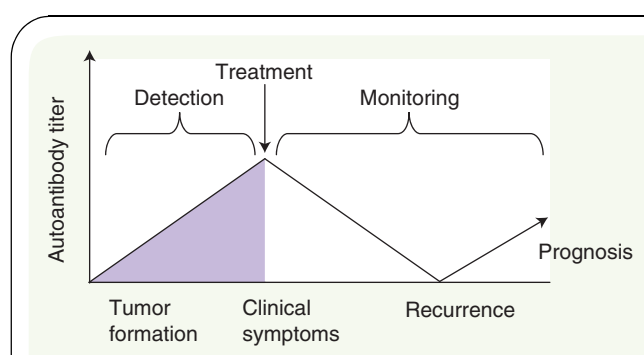


Figure 2. Idealized autoantibody development during cancer progression and treatment. Autoantibodies directed against tumor proteins arise early in tumor development, prior to the onset of clinical symptoms, and could be used for early detection of cancer. Following treatment, autoantibodies represent potential biomarkers for monitoring treatment efficacy. A rise in tumor-specific autoantibodies following treatment may be a useful prognostic indicator. Reproduced with permission from Invitrogen Corporation © 2005.

among others [12–17]. TMAs containing representative sections from multiple patient samples were profiled with affinity purified polyclonal antibodies generated against recombinant protein fragments to derive a proteomic expression profile for more than 20 cancer types [18]. These cancer-specific proteomic signatures may yield potential disease biomarkers. Since biopsied tumor sections are typically formalin fixed in TMA sample preparations, it is important to consider the potential effects of formalin fixation on antigen integrity when evaluating the results of affinity profiling on TMAs.

A microarray-driven expression system has been developed for the parallel functional analysis of multiple gene products. Termed reverse transfected arrays or transfected cell arrays, this technology involves culturing mammalian cells on top of a glass slide that has been printed with discrete complementary (c)DNAs in defined locations in a transfection-compatible solution. Cells growing on the printed areas are transfected with DNA, creating spots of localized protein-specific expression. By profiling the reactivity of antibodies on these arrays, data have been generated demonstrating that tyrosine kinase signaling and protein localization, along with other cellular processes, can be monitored [19]. More recently, RNA interference technology has been adapted to transfected cell arrays to facilitate high-throughput loss-of-function studies [20–22]. In their current format, transfected cell arrays are not appropriate for the discovery of disease biomarkers; however, the ease of automation, combined with the expression of proteins in the context of a mammalian cell, makes this a flexible and attractive platform for validation of discoveries made through alternate assays.

Reverse-phase arrays

Reverse-phase arrays are comprised of whole or fractionated cell lysates, thus allowing the entire proteome of the cell or tissue under study to be sampled. While reverse-phase arrays are not biased with respect to the proteins represented, additional steps,

such as chromatography and mass spectroscopy, are required to identify the actual candidate biomarkers. Reverse-phase arrays have been generated including serial dilutions of the 60 cancer cell line lysates most commonly used for screening compounds for anticancer activity at the National Cancer Institute (NCI). More than 200 antibodies were used to profile relative levels of protein abundance to classify tumor types based on protein expression. Interestingly, when results were compared with gene expression profiling data, the investigators observed good correlation between protein and transcriptional levels for structural proteins, but poor correlation for nonstructural proteins [2]. This finding serves to underscore the importance of proteomic studies as well as the utility of integrating multiple data types. Several laboratories have used reverse-phase arrays to profile the phosphorylation state of cellular proteins that use antibodies both to the protein and to specific phosphorylation sites. These studies were aimed at identifying biomarker candidates and gaining insight into signaling pathways that may be dysregulated in the disease state [23,24]. The potential for discovery using this technology was highlighted by the integration of reverse-phase arrays comprised of cell lysates from microdissected patient tumor biopsied into institutional review board-approved clinical trials at the NCI [23]. Investigators applied high-performance (HP)LC-based sample separation to a colon cancer cell line, generating more than 1700 cell fractions for reverse-phase microarray production. These arrays were assayed with serum from healthy individuals and colon cancer patients in order to establish an immune response signature for colon cancer. Candidate biomarkers identified from immunogenic cell fractions using MS were validated by western blotting [25]. These examples demonstrate that reverse-phase microarrays can provide a discovery-based proteomic approach that is amenable to either antibody-based protein expression profiling or immune response profiling for biomarker discovery. However, the accurate interpretation of the results from studies utilizing antibodies for protein detection requires the antibodies to exhibit a high degree of affinity and specificity for the cognate protein in order to detect low-abundance proteins and to avoid crossreactivity. Multiple studies have shown that data interpretation can be compromised due to unexpected antibody crossreactivities [26–28]. Recent studies have shown that the majority of commercially available antibodies tested exhibit crossreactivity, as demonstrated by interaction with noncognate proteins on protein microarrays [29], indicating a potential limitation of this approach [30].

Forward-phase arrays

By immobilizing purified biomolecules in discrete locations on a solid support, forward-phase arrays narrow the window of proteins sampled relative to reverse-phase arrays, but provide immediate mapping to the antigen interactors observed. Forward-phase arrays have been developed using a diverse panel of biomolecules as interacting partners, including intact antigenic particles, proteins, antibodies, lipids, carbohydrates and both linear and conformationally constrained peptides, including peptides

complexed with major histocompatibility complex tetramers [31–35]. A subset of these protein-detecting arrays, namely antibody arrays, enable quantification of protein levels in complex biologic mixtures. Additionally, arrays have been developed for functional proteomic assays, with which protein–protein interactions or enzymatic activity can be examined.

Antibody capture arrays

Antibody microarrays are made of immobilized immunoglobulins (Igs) or Ig fragments, which capture their analytes in samples of body fluids including serum, cerebrospinal fluid, synovial fluid and alveolar lavage. In one of the first reports demonstrating the feasibility of multianalyte, microarray-based assays, monoclonal antibodies directed against the four human IgG subclasses were arrayed and shown to exhibit subclass-specific recognition of human myeloma proteins [4]. Multiplexed sandwich immunoassays have been developed on arrays, primarily for detection of human cytokines [36–40]. In the most comprehensive study to date, antibody microarrays directed against more than 75 cytokines measured secretion from human dendritic cells in response to lipopolysaccharide or tumor necrosis factor- α induction. Measuring physiologic levels of cytokines requires detection sensitivity beyond the scope of many protein microarray platforms. To address this issue, isothermal rolling-circle signal amplification (RCA) was achieved by coupling to detection antibodies, increasing assay sensitivity and allowing multiplexing due to the reduction in signal diffusion [41]. The commercial availability of highly specific antibodies directed against human cytokines makes them a particularly attractive target for antibody microarray-based assays. In addition, cytokines are secreted proteins that are readily available in biologic fluids, mixtures with many fewer analytes than cell lysates. Of note is the suggestion by MacBeath that only 5% of commercially available antibodies directed against intracellular proteins are suitable for microarray-based analysis of cellular lysates, due to a lack of affinity or specificity, which is in general agreement with published results [30,42]. More recently, MacBeath and colleagues have used antibody capture arrays to monitor activation, uptake and signaling of the ErbB receptor tyrosine kinase family in human tumor cell lines [43]. While these antibodies were assayed for crossreactivity within the ErbB family, they were not examined broadly for off-target binding. Such specificity profiling will be an important subsequent step in the development of antibody array technology. While antibody capture arrays may have demonstrated utility in the evaluation of cellular signaling pathways, the intrinsic constraints on their content arising from the challenge of producing highly specific antibodies could limit their potential as a platform technology for biomarker discovery.

Pathogen & allergen arrays

Circulating antibodies directed against pathogenic antigens have formed the basis for low-throughput clinical diagnostic assays for many years. Diagnostic tests for early detection of

HIV infection, most commonly ELISA-based, monitor blood samples for the presence of antibodies directed against HIV antigens [44]. Assaying blood samples for the presence of circulating antibodies resulting from infection is now possible on microarrays comprised of pathogenic antigens. Microbial antigen microarrays, including single antigens from *Toxoplasma gondii*, rubella virus, cytomegalovirus and herpes simplex virus Types 1 and 2, have been developed that allow simultaneous serologic screening for infection by a panel of common pathogens. These arrays were shown to exhibit assay sensitivities similar to ELISA at a lower cost per analyte [45]. Microarrays containing phage-displayed peptides have been used to assess the HIV-1-specific immune response to 18 peptide mimics of the glycoprotein 41 major immunodominant epitope. These arrays were used to monitor serum antibody reactivity and the immune response profiles before and after the initiation of highly active antiretroviral therapy (HAART). Decreases in fluorescent signals on the arrays were observed for three of five patients following HAART, suggesting either a decrease in the affinity or titer of HIV-1-specific Igs in these patients. The results revealed an assay sensitivity comparable to ELISA, while consuming 1000-fold less antigen and 100-fold less serum antibody [46].

Each of the studies described above focused on the development of microarrays containing a single antigen from each pathogen under study. This concept has been extended to a platform suitable for pathogen biomarker discovery through the development of bacterially expressed protein microarrays comprised of phase-variable proteins from *Neisseria meningitidis*. Expression of phase-variable genes is believed to enhance the ability of *N. meningitidis* to cause an invasive disease, and the arrays included all known phase-variable proteins expressed by this pathogen. Profiling sera from patients suffering from meningitis on the phase-variable protein arrays led to the identification of immunoreactive proteins that have the potential to serve as diagnostic markers of infection or candidates for vaccine development [47]. A whole-proteome pathogenic microarray has been generated using an *Escherichia coli*-based cell-free *in vitro* transcription/translation system to array the 185 proteins comprising the vaccinia virus proteome [48]. While this study clearly capitalized on technological advances in high-throughput cloning and expression to generate whole-proteome microarrays, it is important to consider the technical limitations of the approach taken, including the use of unsequenced PCR products in cloning, which could lead to changes in the protein sequence, as well as the lack of protein purification following *in vitro* expression. Indeed, a significant level of crossreactivity with copurified *E. coli* proteins was observed in the study.

Type I allergy, mediated by IgE, is a hypersensitivity disease affecting more than 25% of the population. Allergy diagnosis is typically performed by provocation testing followed by assaying IgE seroreactivity against allergen extracts. These assays can define allergen sources, but fail to identify specific allergenic biomarker proteins. In an early study, microarrays of a number

of common allergens were used for the detection of multiple allergen-specific IgEs, using RCA technology to enhance detection sensitivity [49]. More recently, over 190 common purified recombinant allergens were microarrayed and used to evaluate patient sera for the presence of allergen-specific IgE. The results of this study demonstrated assay performance similar to established IgE testing formats including the radioallergo sorbent test (a standard technique for measuring allergen-specific IgE antibodies in serum), ELISA and immunoblotting over a range of serum dilutions. The IgE reactivity profiles determined using the allergen arrays were in strong agreement with clinical allergen sensitivity assays [50]. This approach was adapted by Alcocer and colleagues to enable identification of the major structural epitope from the established allergenic protein Ber e 1, which is responsible for eliciting the human allergenic response to Brazil nuts. IgE serum profiling of Brazil nut-allergic subjects identified a conformational epitope responsible for eliciting the Brazil nut-allergic response [51]. While this investigation was not designed to identify novel allergy proteins, it underscores the potential of protein microarrays as a tool for the discovery of native protein biomarkers. These studies illustrate the utility of protein microarrays for identifying proteins as well as specific protein epitopes that elicit an IgE-mediated allergic response, and promise the development of rapid, sensitive and specific allergy screening tools.

Autoimmune response on antigen arrays

Autoantibody production is the hallmark of autoimmune diseases, but the role of autoantibodies in disease development and progression is poorly understood [8]. Due to the unique disease etiology, autoimmunity is ideally suited for immune response profiling on antigen microarrays [52]. Autoantigen microarrays have been developed for performance of large-scale, multiplexed characterization of the autoantibody repertoire from multiple human autoimmune diseases, demonstrating assay sensitivities four- to eightfold higher than ELISA [31]. The challenges associated with whole-proteome analysis have led several investigators to generate microarrays with a limited number of potential antigens represented. This approach was used to study experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis. Arrays comprised of approximately 23 distinct antigens, including overlapping peptides derived from myelin-associated proteins, were profiled with serum from EAE mice. Immune response profiles were used to characterize the evolution of B-cell responses in acute and chronic EAE, and in response to therapy. The results indicated that the diversity of autoreactive B-cell responses in acute EAE is predictive of subsequent disease activity, and that extensive epitope spreading occurs in chronic EAE. These findings suggest that autoimmune responses may be broader than previously believed, a finding that has implications in the development of antigen-specific tolerizing therapies [53]. A subset of the antigenic proteins that give rise to these immune response profiles have the potential to serve as panels of predictive biomarkers.

A focused microarray of 266 distinct antigens has been used to study a mouse model for Type 1 diabetes, in which cyclophosphamide treatment accelerates and synchronizes disease development. Immune response profiling identified a select panel of antigens that allowed predictive discrimination between mice that were sensitive or resistant to cyclophosphamide-accelerated disease (CAD). Interestingly, a distinct autoantibody profile was required for the discrimination of sensitive and resistant populations post-CAD, suggesting that present immunome signatures can predict future disease, and predictive and diagnostic antibody repertoires can differ [54]. While these studies highlight the utility of focused antigen microarrays for autoimmune profiling, the biased antigen set presented on the array makes them unsuitable for the discovery of novel autoimmune biomarkers. High-density microarrays containing more than 2400 purified human proteins have been generated by employing *E. coli*-based expression of a human fetal brain cDNA library, and used in feasibility studies designed to identify novel autoimmune biomarkers [55]. Screening serum from two patients with rheumatoid arthritis and one patient with alopecia areata (a chronic disease leading to rapid hair loss) resulted in the identification of a putative biomarker shared by both diseases [34]. The study of alopecia areata has been expanded using filter-based macroarrays containing more than 37,000 redundant recombinant proteins expressed in *E. coli*. These arrays were profiled with patient serum and used to identify a subset of candidate biomarkers, which were subsequently expressed and used to generate protein microarrays. Screening serum samples from larger patient cohorts led to the identification of a panel of disease-specific biomarker candidates that were validated in western blotting assays [56]. In a separate study, microarrays made from *E. coli*-expressed proteins derived from a mouse T-helper (Th)1 cDNA expression library have recently been used to profile the autoantibody repertoire in serum from a mouse model for systemic lupus erythematosus, resulting in the identification of novel candidate autoimmune biomarkers [57]. It is important to note some of the significant technical issues with the studies mentioned above, including the lack of sequence validation after the reverse transcriptase PCR used to generate the expression clones, and the large fraction of clones that were either not full-length open reading frames or were in the wrong reading frame for expression. In addition, the use of an *E. coli*-based expression system and the fact that proteins were purified under denaturing conditions limit the observations to those interactions that do not require native folding or post-translational processing. Nevertheless, these results highlight the potential of high-content human protein microarrays to facilitate biomarker discovery. Furthermore, many of these issues can be mitigated through newly developed technologies in which full-length proteins are purified under native conditions from insect cells, as discussed later.

Cancer response on antigen arrays

The postgenomic era has seen significant progress in identifying cancer genes that are altered through point mutations, deletions, amplifications or other chromosomal rearrangements.

However, functional investigations of these proteins and their associated signaling pathways suggest that human tumors are heterogeneous diseases of unexpected complexity. Defects in multiple cellular components make disparate contributions to cancer development, influenced by both genetic and environmental factors. Currently, nonmicroarray-based proteomic approaches are the principle high-throughput tools used to experimentally address this complexity. One common method utilizes 2D gel electrophoresis for sample separation and MS for protein identification. This technology has been used to characterize protein abundance in several cancers including neuroblastoma, lung adenocarcinoma, colon cancer, lymphoblastic leukemia, ovarian cancer and hepatocellular carcinoma, resulting in the identification of numerous disease-specific biomarkers [58–62]. Another nonarray-based proteomic approach commonly applied to the search for tumor biomarkers is serologic analysis of cDNA expression libraries (SEREX), in which tumor-derived cDNA libraries are constructed in a phage expression vector. Recombinant proteins are then transferred to membranes and incubated with autologous sera to define the disease-specific immune profile. SEREX has been used successfully to identify tumor autoantigens for a wide variety of tumor types including soft tissue sarcoma, colorectal cancer, renal cancer and leukemia among many others [63–67]. However, SEREX is unlikely to become a routine test for diagnosis and/or prognosis because it is expensive, labor-intensive and requires expertise in a number of sophisticated molecular biologic techniques. In addition, the system suffers from nonspecific absorption of the human serum antibodies to bacterial proteins, which requires a preabsorption step to clear the serum that can cause loss of some tumor-specific antibodies. More importantly, bacterially expressed proteins are frequently misfolded, always unmodified and unprocessed, and lack glycosylation and phosphorylation moieties that are known to be targets of the immune response.

The search for novel cancer biomarkers is now being extended to arrays of purified functional human proteins. Recent advances in high-throughput cloning, expression and purification have led to the production of high-density functional protein microarrays that are suitable for discovery-based proteomic analysis [33]. Yeast proteome microarrays containing approximately 4000 proteins from *Saccharomyces cerevisiae* have been used in affinity-based assays, demonstrating the utility of this assay format for antibody specificity profiling [30]. The authors have recently developed high-density human protein microarrays comprised of approximately 5000 glutathione S-transferase-tagged, full-length proteins affinity purified from insect cells to maximize native folding and post-translational modifications (FIGURE 3). These proteins have been shown to be functional in a variety of assays, including profiling antibody specificity, autoantibody repertoire, protein–protein interactions, small-molecule interactions and assays to identify the substrates of protein kinases [29]. The authors have demonstrated that proteins printed on a nitrocellulose-coated glass slide remain functional for more than 1 year. Additionally, the

authors' results suggest that proteins printed on the nitrocellulose surface are resistant to denaturation by extended incubations in 1% lithium disulfide or heating to over 95°C [DATA NOT SHOWN], which suggests proteins printed on the arrays are extraordinarily stable. In collaboration with researchers at Yale University and the Ludwig Cancer Institute, the authors have compared sera from healthy individuals against sera from melanoma patients, and have identified a number of candidate proteins that are known to be expressed in melanoma cells or to play a role in functional pathways relevant to melanoma progression [UNPUBLISHED DATA]. Efforts to profile additional sera samples and validate a subset of candidate biomarkers are currently underway.

Expert commentary & five-year view

Proteomic analysis of human disease, including allergic reaction, pathogenic infection, autoimmune disease and cancer, has enormous potential to accelerate biomarker discovery. Protein microarrays are perfectly tailored to address the need for rapid, simple, multiplexed assays in order to enable biologic exploration on a proteomic scale. Due to their sensitivity and their ability to simultaneously assay thousands of analytes, protein microarrays are positioned to meet some critically important clinical needs. Protein microarray technology can be used to identify new biomarkers for use in diagnosis, prognosis, therapeutic management, disease monitoring and for vaccine candidates. In addition, this technology is well suited for differentiating clinically distinct populations within a disease group. For example, immunologic profiles could be used to cluster patients according to expected response to a particular treatment, predict treatment-related adverse effects in subpopulations of patients and identify curative antigens in patients with robust anti-disease immune response. A summary of current protein microarray technologies is presented in TABLE 1. Over the next 5 years, protein microarrays are expected to yield discoveries that can be applied in the clinic, as well as opening up new avenues of basic science research.

Protein microarrays represent an emerging technology, but rapid improvements can be envisioned on the horizon. The future of protein microarray technology lies in tens of thousands

of proteins that retain functional activity and native binding interactions. Antibody-capture arrays are likely to benefit from recent advances in the production of highly specific antibodies [18]. Antigen microarrays are currently limited both in the quality and quantity of arrayed features, as well as in their representation of the human proteome. These challenges must be overcome for

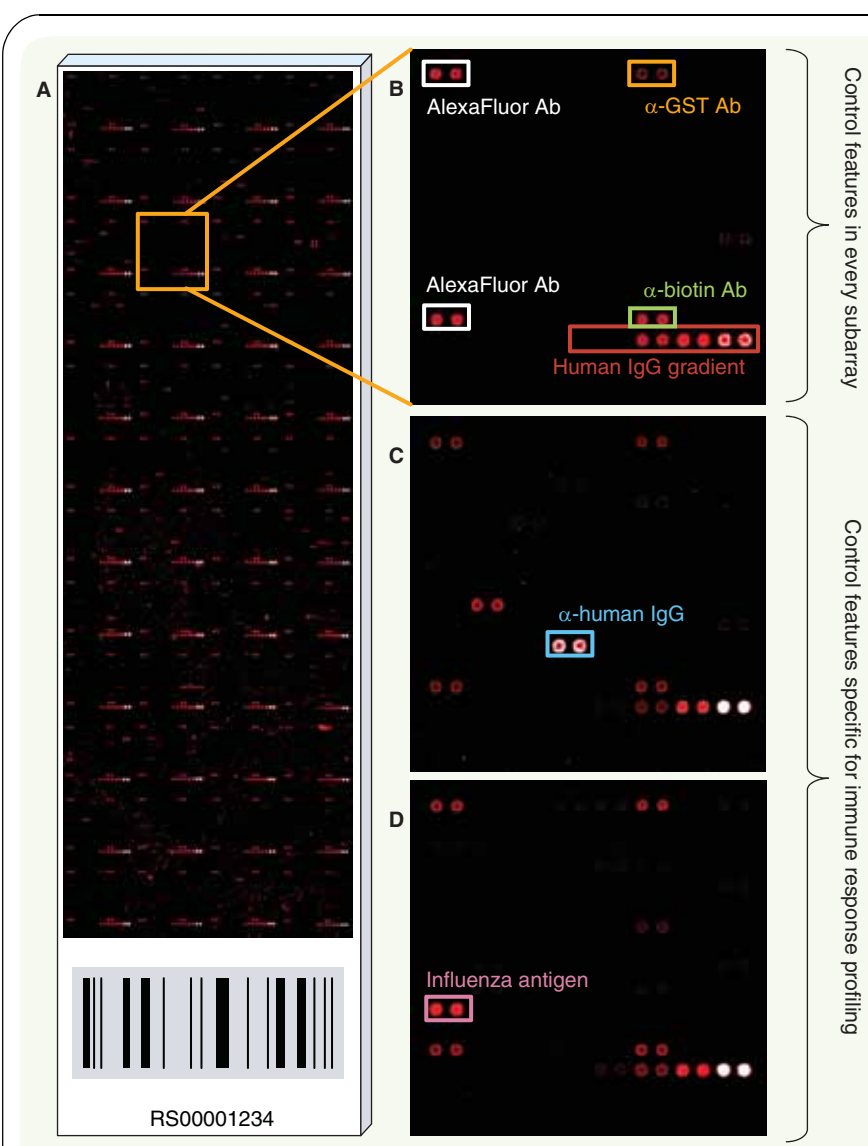
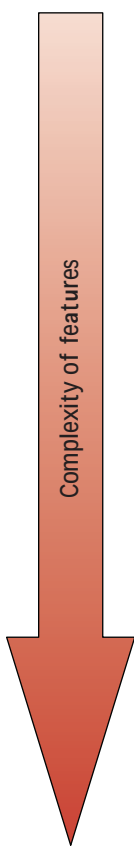


Figure 3. Immune response profiling on human protein microarrays. (A) An array containing approximately 3000 GST-tagged, full-length, recombinant human proteins. (B) One of the 48 subarrays that comprise the full array. Control features contained in every subarray are boxed. Spots of AlexaFluor-labeled antibody (boxed in white) are used to align the grid for data acquisition. The anti-GST and anti-biotin antibodies (boxed in orange and green, respectively) are application-specific control proteins that are bound by the AlexaFluor 647 antihuman IgG antibody used for detection in the human immune response profiling assay. The human IgG gradient (boxed in red) serves as a control for proper performance of the antihuman IgG detection reagent used for immune response profiling. (C & D) Subarrays containing control proteins specific to the immune response profiling application. (C) Antihuman IgG (boxed in blue) binds IgG present in the sample and provides a control for proper performance of the assay. (D) Influenza antigen (boxed in pink) is bound by circulating anti-influenza antibodies in exposed individuals (representing >90% of samples tested to date) and provides an additional assay performance control. Reproduced with permission from Invitrogen Corporation © 2005. Ab: Antibody; GST: Glutathione S-transferase; Ig: Immunoglobulin.

Table 1. Strengths and limitations of protein microarray technologies with potential applications for biomarker discovery.



Array types	Strengths	Limitations	Current applications	Ref.
<i>Forward-phase arrays</i>				
Antibody-capture arrays	Quantitative assay	Limited content (not discovery tool)	Immune response profiling	[36–41,43]
Antigen arrays	High-throughput manufacture, broad applications	Not proteome-scale content	Immune response profiling	[31,33,47,48,50,54–57]
<i>Reverse-phase arrays</i>				
Fractionated cell-lysate RPAs	Proteome-scale content	ID of interactors requires mass spectrometry; biased towards detection of high abundance proteins	Protein expression profiling	[25]
Cell-lysate RPAs	Proteome-scale content	ID of interactors requires mass spectrometry; biased towards detection of high-abundance proteins	Protein expression profiling	[2,23,24]
<i>Whole-cell microarrays</i>				
Tissue microarrays	Proteome-scale content	Sample preparation may compromise antigen integrity	Post-translational modification profiling	[12–17]
Transfected cell arrays	Native proteins in cellular context	Limited content (not discovery tool)	Post-translational modification profiling	[19–22]

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ID: Identification; RPA: Reverse-phase array.

antigen microarrays to provide maximum coverage of the proteome, with array proteins displaying structure–function relationships similar to those observed in living cells. Assay sensitivity represents an additional area of optimization for the microarray platform. Current detection systems most commonly employ chromogenic, fluorometric, radioisotopic or luminescent modalities [23]. Several systems have been utilized to amplify signals derived from low-abundance molecules of interest, including catalyzed reporter deposition technology and RCA [41,68–73]. The next 5 years are expected to bring further improvements in detection sensitivity, such that physiologic levels of serum antibodies and other biomarkers can be readily assayed using a protein microarray format. The properties of microarray surfaces make a significant contribution to the theoretical feature density limit per array, which defines the extent to which proteome-wide screening can be performed in a single experiment [23,74,75]. As seen in the development of DNA microarrays, advances in miniaturization or multiarray sets will quickly overcome the current limit of approximately 10,000 features per array, in scale with the production of whole proteome human protein microarrays, positioning them as true proteomic discovery tools.

Bringing a systems biology approach to the study of human disease represents an important paradigm shift towards global disease profiling. Proteomics has the potential to give a functional assignment to every human protein, including tissue expression profile, subcellular localization and pathway mapping, as well as changes associated with these parameters in the development and progression of disease. Proteomic functional characterization will enable identification of disease biomarkers that will form the foundation of the next generation of human medicine. Panels of disease biomarkers are expected to facilitate earlier diagnosis and more effective treatment of all types of human disease, resulting in a critical shift away from the response-oriented treatment regimens of the past, and a move towards preventative medicine, predictive diagnostics and individualized curative therapies. Realization of this important goal will require integration of multidisciplinary global profiling strategies, a need recognized by the National Institutes of Health [76,77]. Despite the challenges, microarray technology is positioned to make central contributions to this effort, helping to place the goal of personalized medicine within our reach.

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Key issues

- Humoral antibody production is a component of the immune response to invading pathogens, allergens and cancer as well as autoimmune disease. These antibodies represent important biomarkers with potential use in early disease detection, disease staging, prognosis, therapeutic selection and post-treatment disease monitoring.
- Protein microarrays represent an important tool in the burgeoning field of proteomic systems biology and biomarker discovery.
- Protein microarrays appropriate for affinity-based assays can be broadly classified as whole-cell arrays, reverse-phase arrays and forward-phase arrays, each of which are making important contributions to proteomic research.
- Advances in protein microarray technology over the next 5 years are expected to result in the availability of high-density arrays comprised of functional, full-length proteins. The development of these arrays will facilitate disease-specific biomarker discovery with the goal of enabling personalized medicine.

References

Papers of special note have been highlighted as:

- of interest
- of considerable interest

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Affiliations

- Dawn Mattoon, PhD
Scientist, Invitrogen Corporation,
ProtoArray Services, ProtoArray Center,
688 East Main Street
Branford, CT 06405, USA
Tel.: +1 203 848 1147
dawn.mattoon@invitrogen.com
- Gregory Michaud, PhD
Senior Scientist, Invitrogen Corporation,
ProtoArray Support & Applications,
ProtoArray Center, 688 East Main Street
Branford, CT 06405, USA
Tel.: +1 203 848 1135
gregory.michaud@invitrogen.com
- Janie Merkel, PhD
Scientist, Invitrogen Corporation,
ProtoArray Services, ProtoArray Center,
688 East Main Street
Branford, CT 06405, USA
Tel.: +1 203 848 1139
janie.merkel@invitrogen.com
- Barry Schweitzer, PhD
Senior Director, Invitrogen Corporation,
ProtoArray Research & Development &
Operations, ProtoArray Center,
688 East Main Street
Branford, CT 06405, USA
Tel.: +1 203 848 1123
barry.schweitzer@invitrogen.com