

Protein Microarrays See Broader Utilization

Recent Improvements Have Made the Technology More Consistent and Accurate

K. John Morrow Jr., Ph.D.

Protein microarrays are one aspect of highly automated, large-scale biological screening technologies, the other is nucleic acid arrays. As DNA arrays reveal their origin in Southern blotting protocols, protein microarrays reflect their genesis in Western blotting, in which protein mixtures are separated on acrylamide gels, transferred to nitrocellulose, and reacted against specific antibodies.

Protein blots, however, are specialized and labor intensive, highly individualized for characterizing proteins by their molecular weight and other properties. Unlike DNA arrays, much of their success hinges on the availability of quality antibodies that will react strongly and with high specificity against the target proteins. At the recent "PEPTalk" meeting held in California, participants argued the merits of various approaches to building and operating protein microarrays. These include separating, detecting, and characterizing the proteins under study.

Looking from the Opposite Direction

Protein microarray applications run the gauntlet from basic science to drug discovery to diagnostics and personalized medicine, as Antonia Holway, Ph.D., director, microarray applications at Aushon Biosystems (www.

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aushon.com) explained. The company employs a variety of materials and surfaces in its arrays to achieve these goals.

Protein arrays can be implemented in two ways—in the forward-phase mode, the antibodies are printed on the array surface, followed by reaction with the antigen samples; whereas, in reverse-phase mode, antigens are printed, to be followed by reaction of the microarrays with the panel of primary antibodies. With the forward-phase approach, hundreds of antibodies can be printed on a high-content slide for use as a screening tool. This is basically a qualitative assay, demonstrating differences between two samples. Quantitative forward-phase arrays, similar to multiplex ELISAs, are also possible and powerful.

"Reverse-phase arrays can provide high-throughput, multidimensional protein measurements," stated Dr. Holway. In the reverse-phase mode, the antigen, which is printed on the solid substrate, could be any one of a variety of biological materials, including cell lysates, tissue lysates, body fluids, or recombinant proteins. Here, the

investigators can look at hundreds or even thousands of samples on a single slide.

As an example of a study using the reverse-phase microarray approach, Dr. Holway discussed work by Ian Summerhayes of the Lahey Clinic Medical Center (www.lahey.org), whose goal was to identify histone deacetylase inhibitors that can modulate the expression of different tumor and invasive suppressor genes. Summerhayes printed out lysates of nine different bladder carcinoma cell lines treated with histone deacetylase inhibitors. He found that different classes of these agents share similar potential to upregulate the expression of important tumor and invasive suppressor genes involved in bladder tumorigenesis.

In another series of investigations, Benedetta Accordi and Giuseppe Baso of the University of Padua in Italy (www.unipd.it) studied the phosphoproteomic profiles of children affected by B- and T-cell acute lymphoblastic leukemia using reverse-phase arrays, in which they screened the cancer cells for proteins known to be involved in many pathways including cellular proliferation. "Here the goal was to identify key alterations related to disease outcome and drug resistance and even to provide targets for new drugs," Dr. Holway explained.

Yet another application described by Dr. Holway has been developed by 20/20 Gene Systems (www.2020gene.com), and concerns an early detection approach for non-small-cell lung carcinoma. Using a reverse-phase array with phage-expressed proteins as the antigen, a blood test for early detection was developed by measuring the levels of multiple antibodies generated during early stages of the disease. Preliminary results suggest that it may be possible to detect anticarcinoma antibodies in the blood of subjects as much as three to five years before radiographic screening methods are effective.

Dr. Holway concluded her talk by discussing her company's efforts to deal with protein array fabrication challenges. "With our technology we are able to print on a wide range of binding surfaces while handling a variety of buffers and viscosities using extremely low sample volumes," she stated. "This gives us the ability to produce quality arrays in high volume."

Early Detection

"The bottleneck to improving cancer survival rates lies in early diagnosis," said Ruo-Pan Huang, M.D., Ph.D., president of RayBiotech (www.raybiotech.com). Dr. Huang discussed the role of protein and antibody arrays in cancer biomarker discovery, stressing the poor prognosis for late-stage ovarian cancer. He bemoaned the fact that tremendous effort has been expended in the search for individual, cancer-specific markers with few positive results.



Aushon Biosystems' 2470 Arrayer is a high-throughput microarrayer designed to provide rapid, high-quality printing of a wide variety of materials, including cell lysates, onto most any substrate. Substrate platens such as the microscope slide platens in this photo are housed in the substrate elevator and printed sequentially, allowing for walk-away automation.

Indeed, a list of 1,261 proteins believed to be differentially expressed in human cancers have been compiled by researchers at the Plasma Proteome Institute (www.plasmaproteome.org), yet only nine have made the epic voyage to final FDA approval as tumor-associated antigens. Many workers in the field now believe that the search for individual cancer biomarkers will be fruitless, and multiple biomarkers will be required to identify cancers in screening protocols.

Dr. Huang's group has designed immunoarrays in which antibodies are carefully printed onto substrates in order to optimize sensitivity and dynamic range, and then subjected to performance testing. The arrays were based on cytokine proteomics, recognizing the role that cytokines are known to play in a number of disease processes. In fact, cytokines are the most widely studied of all protein families. The arrays can detect more than 500 cytokines in one experiment in the picogram range with high specificity and little cross-reactivity. They have proven easy and cost-effective to use.

Dr. Huang and his collaborators have used RayBio® (www.raybiotech.com) Cytokine Antibody Arrays to measure the presence of 174 different cytokines in a sample of malignant ovarian tumors, benign tumor material, and normal tissue. The data was analyzed using a neural network procedure, a statistical data-modeling tool that permits classification of the different markers according to their predictive power. The results demonstrated that the antibody array analysis had a high agreement (between 60 and 90%) with the standard criteria derived from clinical diagnosis.

See Protein Microarrays on page 32

News Genomics & Proteomics

Florgenex and the National Center for Genome Resources Form Partnership

The National Center for Genome Resources (www.ncgr.org) established a services partnership with Florgenex (www.florgenex.com). The JV will combine Florgenex' genomic discovery and application tools with National Center for Genome Resources' bioinformatics and sequencing capabilities.

Nanosys and Harvard License Vista Therapeutics Nanowire Technology

Vista Therapeutics (www.vistatherapeutics.org) has signed license agreements with both Nanosys (www.nanosysinc.com) and Harvard University (www.harvard.edu) involving patents and patent applications regarding nanomaterials, nanotechnology, and the employment of nanowire-based field effect transistors as biosensors.

Harvard and Nanosys gained an equity

position in Vista, and also up-front license and downstream royalty payments. Vista set up exclusive, worldwide rights to use nanowires to detect biomarkers associated with organ or tissue damage, and any form of treatment or therapeutics-associated adverse response.

PROACTIVE Given \$3.8M for Plasma Biomarker Infrastructure

The European Commission 7th Framework Program granted €3M (\$3.8 million) to the new consortium PROACTIVE to develop a high-throughput plasma biomarker research platform.

The infrastructure will be executed as a series of pilot biomarker projects designed to improve colorectal cancer detection. Olink Bioscience (www.olink.com) will coordinate the three-year project, which will cultivate methods and reagents for highly multiplexed proximity ligation assays for protein detection. The project will also develop data-management tools and tailored statistics. n

Protein Microarrays Continued from page 30

“We concluded that protein and antibody arrays are a reliable tool for biomarker discovery,” Dr. Huang continued. “Unique expression patterns using protein and antibody arrays can be used to predict ovarian cancer with high accuracy. It is our hope that further study may lead to identification of clinically useful biomarkers for early detection of this disease. In addition, our platform can also be easily applied to other biomarker discovery programs.”

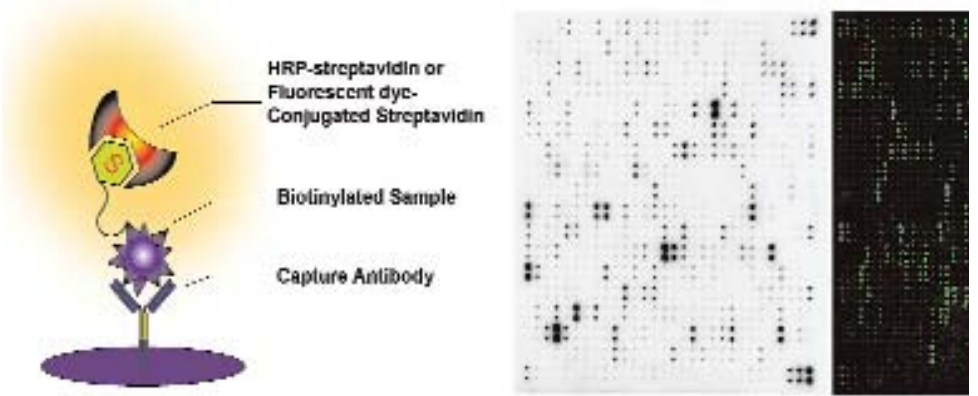
Colorimetric Detection

Bryce Nelson, Ph.D., vp, R&D at Gentel Biosciences (www.gentelbio.com), discussed the need for a workable and affordable multiplexed protein detection

system designed around a traditional ELISA format.

“Our focus is to make protein arrays accessible to laboratories currently using ELISA technology,” Dr. Nelson stated. Gentel explores the familiar goal of disease biomarker discovery aimed toward validation through clinical trials using nitrocellulose surface platforms. Such markers could monitor therapeutic interventions and identify new therapeutic targets.

The microarrays are evaluated using either a fluorescence-based or a colorimetric-based assay system. For fluorescent determination Gentel uses PATH® Protein Microarray Slides, composed of transparent nitrocellulose on glass substrates and



RayBiotech reports that its Human Biotin-Labeled-Based Antibody Arrays can simultaneously detect expression levels of 507 human proteins.

formatted in standard microarray slide and 96-well format. Alternatively, for colorimetric determination, the APiX™ Protein Microarray Slides use transparent nitrocellulose on plastic or glass substrates.

The system is compatible with Gentel's forthcoming APiX Colorimetric Reader and detection reagents, and both systems can be automated. Subsequent investigations demonstrated that the colorimetric assay system is quite sensitive, can multiplex several hundred protein in a well, and can read plates in minutes by a simple, low cost reader, Dr. Nelson reported.

The APiX colorimetric system uses a gold particle-enhanced silver deposition technology that is “basically a silver stain for microarrays. We have shown that the system yields higher sensitivity than bead-based and fluorescence-based microarrays,” he added.

Gentel has used the APiX colorimetric system in conjunction with antibody array methods to screen serum protein glycosylation changes in hundreds of proteins as potential cancer biomarkers. The APiX GlycoBiomarker array kit, designed for the characterization of disease-associated glycan alterations, is used for the identification of new biomarkers and the analysis of factors that regulate glycan structures.

Dr. Nelson and his colleagues at Gentel have also validated the performance of the APiX colorimetric system using a number of different array formats, including Eprogen's (www.eprogen.com) multidimensional liquid-phase protein fractionation system. “As these technologies mature, there will be a need for an affordable multiplex protein detection format for diagnostics that doesn't sacrifice performance,” Dr. Nelson added. “We believe the features of our gold particle-enhanced silver deposition platform move us closer to this goal.”

Diagnosis of Rheumatoid Arthritis

“Antibodies to cyclic citrullinated peptide may precede the onset of rheumatoid arthritis by as much as ten years,” stated William Robinson, M.D., Ph.D., assistant professor of medicine in the department of immunology and rheumatology at Stanford University (www.stanford.edu).

Current treatments for the disease include small molecules such as methotrexate, hydroxychloroquine, and biologicals such as

anti-TNF and anti-CD20 recombinant antibodies. Dr. Robinson's team is developing protein arrays to ferret out new markers. “Through profiling of circulating autoantibodies and cytokines, we have identified markers with predictive utility,” he continued.

The current model of the chain of events leading to the disease ties together an adverse combination of genetic markers and potential environmental inputs, including smoking, hormones, and infections, setting the stage for the early, preclinical forms of the disease. Moreover, there are subtypes of the disease that respond differentially to the available therapies.

Part of the pathogenesis of rheumatoid arthritis may reside in citrullinated proteins that are produced by enzymatic deimination of arginine residues in proteins. This increased enzymatic activity results in an unfolding of proteins by loss of a positive charge in arginine residues, triggering an autoantigenicity cascade. This pernicious series of unfortunate events takes place in the synovial fluid within the joint cavity leading to lymphocyte invasion, production of TNF and other pro-inflammatory cytokines, production of degradative enzymes, and ultimately joint destruction.

Dr. Robinson's team designed protein arrays in which a large coterie of antigens were printed onto microplates and then probed with patients' antisera. Historical experience in the management of patients speaks loud and clear: early intervention with at-risk patients resulted in 56% remission. A retrospective analysis of serum samples taken from patients who later developed arthritis demonstrated that many cytokines and autoantibodies appeared prior to the onset of clinical disease, yet far and away the best prognosticator was antibodies to cyclic citrullinated peptide.

Profiling Serum Antibodies

Serametrix (www.serametrix.com) has developed assays for multiplex detection of serum antibodies using an array of tumor antigens, according to Henry Hepburn-Scott, Ph.D., vp, business development. These antibodies are raised by patients, so the approach is similar to the Aushon platform. The antibodies were screened against proteins that Dr. Hepburn-Scott and his colleagues had select-

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ed according to the criteria that all were associated with cancerous phenotypes, had demonstrated immunogenicity, and could serve as biomarkers for drug discovery and development. Dr. Hepburn-Scott and his colleagues used this strategy to detect tumor antigens in a cohort of 16 different patients.

Data from such studies can help to monitor immune response to drugs, give early warning of immune-related adverse events, and even provide a high-value companion diagnostic to identify responders in pre-treatment cohorts.

2-D Protein Biochips

“We have focused our 2-D protein fractionation platform on several different cancers using reverse-phase microarrays to look for new and more specific protein biomarkers using patient sera,” stated Timothy J. Barder, Ph.D., president of Eprogen.

Dr. Barder described Eprogen’s approach to biomarker discovery and validation as a two-stage process: first, applying the 2-D all-liquid phase fractionation strategy based on HPLC to solve the complex intact protein-expression analysis problem; and second, extending these fractionation strategies to produce 1-D and 2-D reverse-phase protein microarrays for probing with antibodies or autoantibodies.

To facilitate this separation technology, Dr. Barder’s team employs NPS® (nonporous silica) in an ultrafast, HPLC support for improved speed and resolution, tailored for proteins and mass spectrometry (MS). It provides a separation that is far superior to traditional methods such as 1-D gels, he reported.

Combining 2-D liquid-phase HPLC with microarray technologies opens up a range of possible strategies including screening multiple arrays produced from the same tissues with many different antibodies, or by analysis of autoantibody signatures or profiles (serological assay) for monitoring disease-progression analysis. This approach can also be applied to biomarker discovery and drug development studies, probing the 2-D arrays with well-documented patient serum samples.

Dr. Barder discussed the use of this approach in the investigation of prostate cancer biomarkers. David M. Lubman and his associates at the University of Michigan Medical School used a multidimensional liquid-phase protein fractionation of localized and metastatic prostate cancer tissue lysates to build protein microarrays, which were tested against serum from a cohort of 34 patients with either benign prostatic hyperplasia or clinically localized prostate cancer.

Spots on the microarrays that reacted positively were isolated and analyzed by MS. A number of proteins were detected that reacted against the autoantibodies from cancer patients’ serum, including 29 that were found to be prostate cancer specific; 12 of these were identified by single peptides. These may be proteins involved in pathway dysregulation that might otherwise be suppressed by the complexity of

the cancer proteome. A larger melanoma study is currently under way using this approach to look for new serum markers for monitoring this cancer.

“The 2-D gel-drop microarrays can be highly automated, allowing maximum information from a given sample,” Dr. Barder concluded. “Many microarrays can be derived from a single fractionation and made available for testing with a variety of probes and detection methods.”

While protein microarrays are an engaging technology, they still present technical problems that may disarm the unwary. Their performance is dependent upon the antibodies used in the detection system, and conditions must be carefully optimized to ensure proper binding, and to eliminate nonspecific interactions. It should be cautioned that many antibodies don’t work well as capture reagents and bind poorly when reacted against cell extracts, even though

they perform well under denaturing conditions and in Western blotting protocols.

There is a heightened awareness of these limitations within the biotech community, and the quality of data generated from protein microarrays is much more consistent and accurate than in the early days of this technology. These improvements should help to lower the high attrition rates of biomarker evaluation and allow the development of a new generation of biomarkers. GEN



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