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Applications of antibody array platforms

Brian B Haab

Antibody arrays are valuable for the parallel analysis of multiple proteins in small sample volumes. The earliest and most widely used application of antibody arrays has been to measure multiple protein abundances, using sandwich assays and label-based assays, for biomarker discovery and biological studies. Modifications to these assays have led to studies profiling specific protein post-translational modifications. Additional novel uses include profiling enzyme activities and protein cell-surface expression. Finally, array-based antibody platforms are being used to assist the development and characterization of antibodies. Continued progress in the technology will surely lead to extensions of these applications and the development of new ways of using the methods.

Addresses
Van Andel Research Institute, 333 Bostwick Grand Rapids, MI 49503, USA

Corresponding author: Haab, Brian B (brian.haab@vai.org)

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Introduction

Antibody-based measurement technologies have long provided an important tool to detect and manipulate specific biological molecules. Whereas previous uses of antibodies and related affinity reagents focused on single targets in each assay, recent developments have enabled the multiplexed use of antibodies in arrays so that many targets can be measured in parallel, sometimes in very small sample volumes. The uses of such arrays are varied and new applications and formats continue to evolve.

The ability to measure multiple proteins in parallel has many benefits. Significant savings per assay in time, cost, and sample consumption are possible, especially if the assays are miniaturized. For example, conventional immunoassay formats such as enzyme-linked immunosorbent assays (ELISA) can consume 50–100 μl of a sample per protein target, using 500 ng of capture antibody. The measurement of 10 different proteins with such a format, even without duplicates, could consume a stock supply of a clinical sample. By contrast, an array format could measure dozens of proteins in a total sample volume of just 10 μl, using just 0.2 ng of each capture antibody per sample. Clearly the opportunity for multiprotein studies is made feasible by such a strategy. Aside from the practical benefits, parallel measurements have scientific use. As proteins interact in networks and have overlapping or complementary functions, it might be necessary to look at the coordinate levels of multiple proteins in a highly controlled experiment to get a meaningful picture of a biological state. In studies to identify or characterize biomarkers, it is useful to efficiently test many candidate markers or to examine the coordinated changes in panels of markers and the potential use of multiple markers together.

This review covers the ways that variants of antibody array technologies have been, and are being, applied. Optimization studies and comparisons of technologies are covered in greater detail in other reviews [1–4,5••]. In addition, this review is focused on affinity arrays designed to capture and analyze specific proteins, rather than on protein arrays designed to test biological interactions, which are reviewed elsewhere [3,6–9]. Most published applications have used a planar format, in which antibodies are spotted side-by-side on a flat surface, but similar applications using a bead-based method with flow cytometry readout have also been reported. Studies using conventional monoclonal and polyclonal antibodies have dominated the literature, but recombinant affinity reagent sources have also appeared and presumably would be compatible with any of the formats. As this review focuses on applications rather than on demonstration or proof-of-principle experiments, the emphasis is on methods using conventional antibodies.

Applications of parallel measurements of protein abundances

After it was established that miniaturized arrays of antibodies could be used to efficiently assay multiple proteins [10,11], the earliest and most numerous applications of the technology have involved measuring the abundances of multiple proteins in sets of samples, usually to discover associations with disease or new biomarkers. Biomarker discovery applications would ideally involve screening proteins on a proteome-wide scale, so that all possible biomarkers or disease-associated proteins would be probed. Current affinity-array technologies cannot begin to approach that level of screening, however, partly because antibodies or other affinity reagents to target all expressed proteins are not available, and partly because developing and validating so many immunoassays into a single tool would be prohibitively labor-intensive. Furthermore, the presence of splice variants and

modifications multiplies the number of unique protein species, making the concept of affinity-based profiling of proteome-wide abundances even more unapproachable. Because of these limitations on the number of proteins that can effectively be measured in parallel, efforts to profile protein abundances using antibody arrays have focused on specific, targeted sets of proteins.

Multiplexed sandwich assays

Cytokines are an important group of proteins that have been studied using antibody arrays. These signaling factors reside in extracellular fluids and, as they can arise from many different cell types, information on their abundance cannot be accessed using RNA expression studies. Thus, protein analysis methods are important for this application and, because a comprehensive view of cytokine levels would be more valuable than measuring one at a time, antibody array methods could be especially valuable.

Most array-based strategies for measuring cytokines have used multiplexed sandwich assays, in which a cocktail of detection antibodies, each antibody corresponding to one of the arrayed capture antibodies, is used to detect the levels of the captured target proteins. The detection antibodies are often biotinylated, so that the amounts bound to the array can be measured using streptavidin labeled with a fluorophore or an enzyme for subsequent readout. Sandwich assays can be highly sensitive and specific, but in a multiplexed setting great care must be taken in the development of the assays to ensure no cross-reactivity or interference between the detection antibodies. Also, some development may be required to find a matched pair of antibodies that work well together for each target. Systematic methods for multiplexed assay development and quality control have been described [12•]. Developing non-interacting sets of sandwich assays becomes extremely difficult after multiplexing a few dozen, and most developers choose to segregate arrays after that point (e.g. making two 25-assay arrays instead of one 50-assay array).

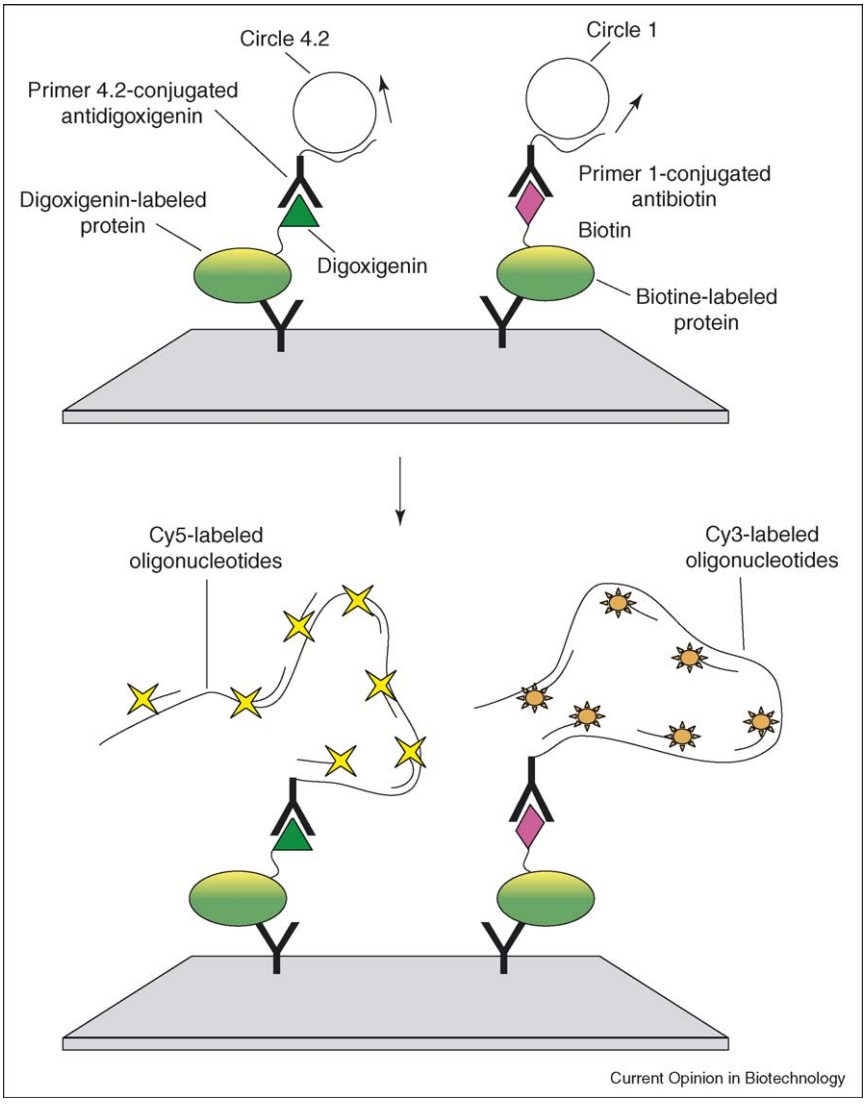
The value of multiplexed cytokine measurements was demonstrated in several studies using detection with rolling-circle amplification (RCA). RCA can greatly enhance fluorescence signals over non-amplified detection [13–15], enabling the detection of low-abundance proteins like cytokines. A schematic representation of a two-color version of the method (described below) is shown in Figure 1. In an early demonstration of the technology, secretions from human dendritic cells were studied after stimulation with lipopolysaccharide or tumor necrosis factor α (TNF-α), showing previously known and novel inflammatory cytokine inductions at various time points [14]. Another study aimed to identify serum markers for the early detection of ovarian cancer [16]. Some 169 cytokines and other proteins were examined in the sera of women with ovarian cancer, recurrent ovarian cancer, and no disease. Initial experiments defined a panel of four proteins, leptin, prolactin, osteopontin and insulin-like growth factor-II, which together could distinguish the cancer from control samples. The testing of that panel on blinded samples distinguished ovarian cancer patient samples from control patient samples with 95% sensitivity and 95% specificity. This test still needs to be evaluated using larger sample sets and samples from other types of disease. Likewise, 78 different cytokines, growth factors and soluble receptors were profiled in the sera of patients with inflammatory bowel disease, comparing patients with active disease to those in remission [17]. Certain cytokines were repressed in the patients with active disease. A study of cord blood found that certain inflammatory cytokines characterize infants with cerebral palsy. These factors could be involved in the inflammation and brain damage that leads to cerebral palsy [18].

A similar cytokine array platform was applied to the analysis of some less commonly studied bodily fluids where protein levels cannot be estimated using transcript profiling. For example, tear fluid samples, collected with capillary tubes from closed or open eyes or upon nasal stimulation, were analyzed to catalog the presence of specific cytokines, chemokines and growth factors [19]. Likewise, cerebral spinal fluid samples were examined to identify cytokines that could be associated with infectious meningitis [20]. Tumor interstitial fluid was also analyzed using cytokine-specific antibody microarrays [19]. Tumor interstitial fluid perfuses the tumor microenvironment and its analysis can give information about paracrine and autocrine mediators that are important in cancer. All these studies used a commercially available array designed on the basis of earlier work, in which antibodies arrayed onto membranes were detected with a cocktail of anticytokine antibodies and developed with electro-chemiluminescence (ECL) [21]. A similar antibody microarray using ECL detection of five different angiogenic factors found the levels of these factors to be higher in the sera of ovarian and endometrial cancer patients than in controls [17]. A related study measured the levels of cytokine secretions from cultured breast cancer cells to show an association between interleukin-8 levels and invasiveness [18]. These results show the capability of array-based, sandwich-immunoassay technologies to profile variation in low-abundance proteins and to discover associations with disease. The discovery of powerful biomarkers using these methods has yet to be reported, perhaps reflecting the difficulty of that goal, but the ability to capture information from low-abundance proteins in a low-volume and multiplexed format is nevertheless valuable for many other applications.

Label-based arrays

A complementary technology to sandwich assays is label-based detection, which has also been used on arrays to profile the abundances of multiple proteins. In label-

Figure 1



Schematic representation of two-color RCA on antibody microarrays. Two pools of proteins are respectively labeled with digoxigenin (green triangle) and biotin (pink diamond). Primer 4.2-conjugated antidigoxigenin and primer 1-conjugated antibiotin bind to the captured proteins, followed by hybridization of circle 4.2 and circle 1. Polymerase extends the primers using the circles as templates. Cy5-labeled oligonucleotides, complementary to the extended DNA from primer 4.2, and Cy3-labeled oligonucleotides, complementary to the extended DNA from primer 1, are then hybridized to the extended DNA strands, producing signal amplification in two colors.

based detection, all proteins in a sample are labeled with tags, such as biotin or the fluorescent dyes Cy3 and Cy5, that allow detection after capture on an antibody array. Only one antibody per protein target is required, as opposed to a matched pair for the sandwich assays, so it is easier to acquire the antibodies and develop the assays. As cross-reactivity between detection antibodies is not a concern, the level of multiplexing is not hindered. Another advantage is the ability to label different samples with different tags in a two-color assay, so that test samples can be mixed with reference samples. Having a reference sample enables normalization methods that improve precision [22]. A two-color assay is also competitive, meaning that proteins from the test sample compete with proteins from the reference sample for binding sites on the immobilized antibodies. Competitive assays have experimental advantages over one-color, non-competitive assays, such as the ability to measure a broader range of concentrations at a single sample dilution [23]. Nevertheless, the label-based method also has drawbacks relative to sandwich assays. The use of just one antibody per target means that specificity can be lower and, as all proteins are labeled, backgrounds and detection limits can be higher. The approach is probably best used as a practical method to screen binding to many antibodies targeting a wide variety of proteins.

Whereas sandwich-assay arrays have mainly targeted cytokines and related secreted signaling molecules, label-based arrays have targeted a wider variety of proteins covering a range of concentrations, functions and tissue locations. Serum proteins were labeled with Cy3 and Cy5 and profiled on antibody arrays to identify potential biomarkers for prostate cancer [24] and cystic fibrosis [25]. Several proteins that differed between cases and controls were identified in each study. Proteins in cell-culture systems were also labeled with Cy3 and Cy5 and profiled to study the effects of radiation [26] and angiogenin stimulation [27] on protein expression. In a different approach, proteins secreted into the media of culture cells were labeled with biotin, captured on antibody arrays, and detected with Cy3- or Cy5-labeled streptavidin [28]. This system was able to detect cytokine secretion associated with estrogen receptor status in breast cancer cells.

Label-based detection has also been used to look at protein expression in human tissue samples. Laser capture microdissection was used to remove selected regions of tumor and stroma from a frozen squamous-cell carcinoma tissue sample [29]. The isolated proteins were labeled with biotin, applied to antibody microarrays on nitrocellulose, and measured with chemiluminescence. The authors identified proteins in both the tumor tissue and the surrounding stroma that had levels correlating with advancement of disease. In another study using resected tumor tissue [21], proteins isolated from 30 different hepatocellular carcinoma tumors and from 15 normal liver specimens were analyzed using Cy3 and Cy5 labeling on microarrays containing 83 different antibodies. In total, 32 of the proteins exhibited differential expression between the tumor and normal groups, and the levels of many of the proteins were confirmed by western immunoblot techniques. A similar study used a commercially available antibody microarray targeting 378 different proteins to compare protein levels between malignant and normal breast tissue from the same patient [22]. Several proteins were found to have higher levels in the malignant tissue, and the levels were confirmed by immunohistochemistry.

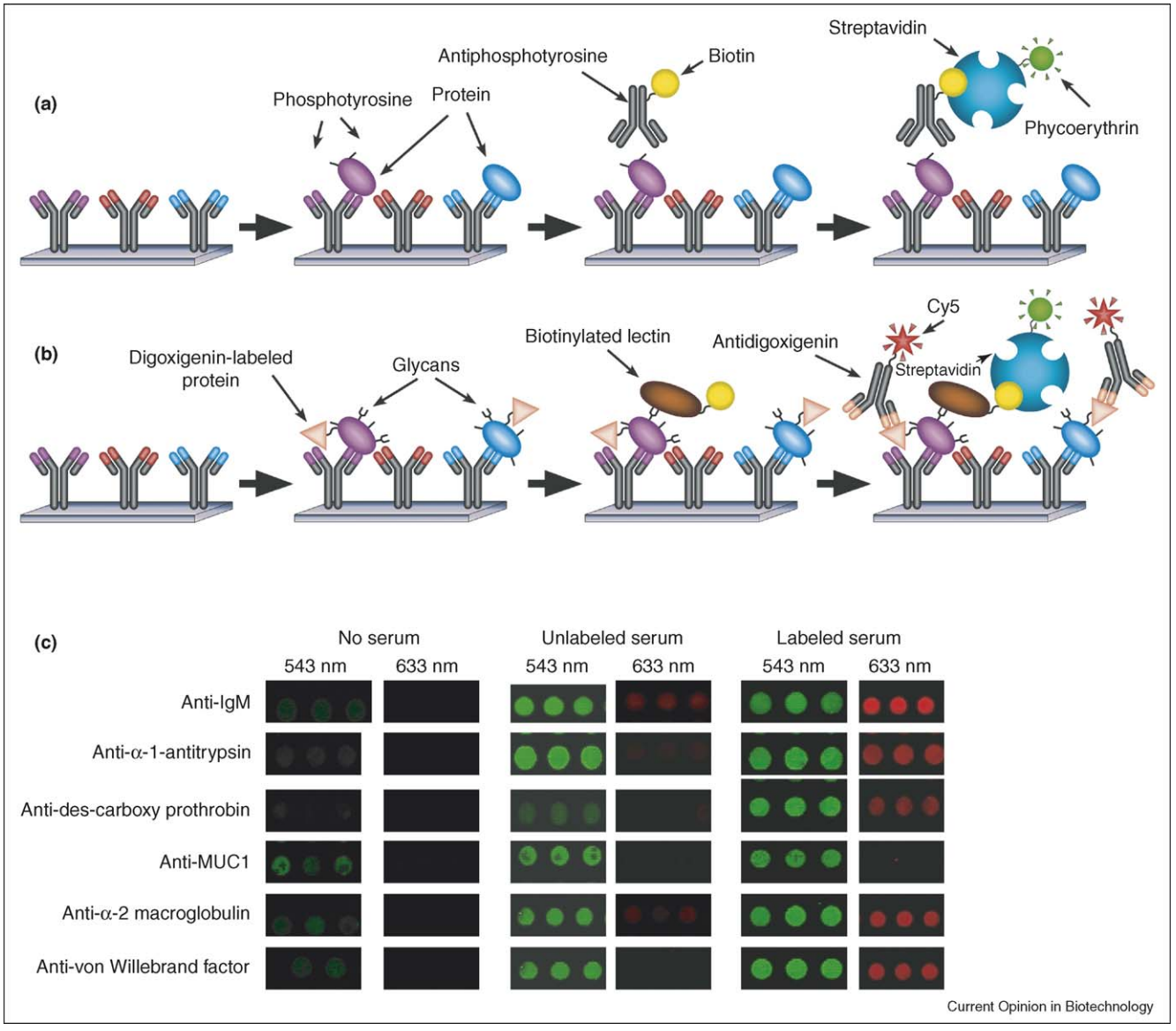
In an effort to lower the detection limits of the two-color, label-based strategy, a method was developed to amplify the fluorescence from the labeled proteins using rolling circle amplification (RCA) [15]. Serum proteins that had been labeled with either biotin or digoxigenin were captured on antibody arrays. RCA producing green fluorescence was used to detect the biotin tag, and RCA producing red fluorescence was used to detect the digoxigenin tag. This method, called two-color RCA (TC-RCA), produced much higher fluorescence than the method described above and also enabled the detection of lower abundance proteins. The TC-RCA method was applied to serum biomarker discovery for lung and pancreatic cancers [30,31•]. In the pancreatic cancer study, profiling binding to 92 antibodies using samples from 142 patients revealed multiple proteins associated with both malignant and benign pancreatic disease, some of which were previously unknown to be associated with pancreatic cancer. The study further showed that the use of multiple, different measurements together could improve diagnostic accuracy over individual measurements.

Measuring post-translational modifications on arrays

The biological roles of proteins are determined not just by their abundance, but also by modifications and interactions that occur after translation such as phosphorylation, glycosylation, cleavage, oxidation or reduction, and lipid attachment. As this level of protein information cannot be probed using DNA- or RNA-based methods, protein array and antibody array methods are a natural platform for such studies. A novel application of antibody arrays has been to detect certain post-translational modifications on multiple proteins. One strategy for this purpose employs a modified sandwich assay in which the detection antibody targets a particular modification instead of the core protein. Schematic representations of 'heterogeneous' sandwich assays are shown in Figure 2 for the measurement of phosphorylation and glycosylation states.

Protein phosphorylation is an especially important regulator of many processes inside cells. As complex signaling networks can be regulated by multiple coordinated phosphorylation levels, the ability to probe phosphorylation states of multiple proteins in parallel could offer new insights into signaling processes. In studies of the signaling networks of interacting proteins [32], proteins were captured by immobilized antibodies and were probed with antibodies targeting either the proteins themselves or phosphotyrosine. By looking at the ratio of phosphotyrosine to total protein, the level of activation of the proteins could be measured. In this way, the authors quantified epidermal growth factor receptor and ErbB2 activation upon stimulation with epidermal growth factor and treatment with a tyrosine kinase inhibitor. The extension of this technique to the measurement of 20 or more members of particular signaling pathways would allow the systematic analysis of signal transduction systems under a variety of conditions. Another report demonstrated the use of antibody arrays to detect changes in the phosphorylation state of multiple proteins [33••]. Cell extracts were incubated on arrays of antibodies targeting various cellular components of signaling pathways, and the bound proteins were probed with labeled antiphosphotyrosine antibodies. Changes in the phosphorylation levels as a function of drug and growth factor treatment were measured on the microarrays and validated by mass spectrometric analyses. A related approach to measure protein modifications using antibody arrays was adopted by Ivanov and colleagues [34]. In their study, whole-cell extracts were immunoprecipitated using

Figure 2



The detection of phosphorylation and glycosylation on antibody arrays. **(a)** Phosphorylated protein detection. Proteins are captured by antibody arrays and the arrays probed with biotinylated antiphosphotyrosine. Bound antibody is then detected using fluorescence from streptavidin-phycoerythrin, which binds to biotin. **(b)** Two-color detection of glycans and proteins using digoxigenin-labeled proteins. Proteins are labeled with digoxigenin and captured on antibody arrays. The glycans on the proteins are detected with biotinylated lectins, which subsequently bind streptavidin–phycoerythrin, and the protein levels detected using Cy5-labeled antidigoxigenin. **(c)** Demonstration of glycan and protein detection on antibody arrays. Antibody arrays were incubated with no serum, unlabeled serum or digoxigenin-labeled serum. The arrays were then incubated with biotinylated SNA lectin, followed by detection with Cy5-labeled antidigoxigenin (red, 633 nm) and Cy3-labeled antibiotin (green, 543 nm). Fluorescence from the spots is shown in both color channels from the indicated antibodies.

antibodies targeting phosphotyrosine, ubiquitin or acetyl lysine, and the precipitates were fluorescently labeled and applied to antibody microarrays. The level of binding to each antibody reflected the level of modification of each target protein.

Another related approach has been developed in the author's laboratory for the measurement of specific glycans on proteins captured by antibody arrays (Figure 2b). Changes in specific glycan structures have important roles in disease, and the ability to efficiently profile those changes on many different, specific proteins would be valuable in characterizing glycan regulation or associations with disease. After specific proteins from biological samples are captured by the immobilized antibodies, a biotinylated lectin is used to bind glycans on the captured

proteins. Streptavidin linked to the fluorescent molecule phycoerythrin is then used for detection. Lectins are plant and animal proteins with natural carbohydrate-binding affinity. Each lectin type has specificity for a particular glycan structure, so the glycan structure measured on the capture proteins can be specified by the lectin used for detection. To measure both protein level and glycan level in the same experiment, the proteins can be tagged to allow detection with a different color fluorophore than that used to detect the glycan (Figure 2b). The detection of both proteins and glycans on antibody arrays was confirmed after incubation of either buffer, unlabeled serum or labeled serum on antibody arrays, followed by detection with the biotinylated lectin *Sambucus nigra* (SNA) and Cy5-labeled antidigoxigenin antibody (Figure 2c). This method is currently being used to profile the variation in glycan levels on serum proteins in cancer patients. Such studies might reveal novel cancer markers or could identify glycan alterations involved in the cancer process.

Other formats and applications

Antibody-array-based methods have been applied in other novel ways to obtain additional levels of information about proteins. Most biological processes are driven at some level by enzymes, and the states of those processes are determined by the activity levels of the enzymes. An antibody microarray method was developed to profile enzyme activities in complex proteomes [35•]. Complex protein samples were treated with fluorescent activity-based probes, which bind to the active sites of particular classes of enzymes, and the labeled enzymes captured and detected on antibody microarrays targeting those enzymes. Compared with corresponding gel-based methods, the microarray platform improved the sensitivity and specificity and reduced sample consumption. Using this approach, the activities of prostate-specific antigen, urokinase, tissue plasminogen activator, and matrix metalloproteinase 9 were measured in two breast cancer cell lines, with and without the addition of protease inhibitors.

Another type of information that can be gathered from antibody arrays is the expression levels of cell-surface proteins on whole cells, without protein isolation [36]. Suspensions of cells were incubated on antibody microarrays, and the amount of cells bound to each antibody quantified by dark field microscopy. The arrays specifically targeted membrane-bound 'CD' antigens, which define the phenotypes of leukocytes. The authors speculated that the characterization of the spectrum of CD antigens on leukocytes might define certain disease states. This principle was confirmed in applications of the method to identify CD antigens on leukocytes that discriminated chronic lymphocytic leukemia patients from control patients [36], in the identification of drug-induced changes in cell-surface antigens [37], and identifying changes associated with HIV infection [38]. Recently, the technique was use to look at surface molecules of colorectal cancer cells taken directly from tumors [39••]. As a mixture of cell types were present in the suspensions, the arrays were probed with fluorescent-dye-labeled antibodies targeting a tumor marker, so that the tumor cells could be distinguished from the other cells that bound to the arrays. This modification to the technique is important because now the immunophenotypes can be obtained from cell subpopulations that are the minority of a tissue specimen. Certain CD antigens were clearly upregulated specifically on the surfaces of the tumor cells, which might have implications for the phenotype of those cells or for the definition of cancer drug targets.

Conclusions

The types of applications and platforms of antibody arrays are likely to continue to evolve in the future. The continued development of commercial products and custom services for array-based immunoassays should also broaden the usage of these technologies. These tools could be especially useful to complement and support other proteomics technologies; for example, studies to identify new protein expression patterns or forms using mass spectrometry could be followed by highly multiplexed affinity methods for efficient validation. One can envision many situations where it will be necessary to have a practical means to obtain multiple protein measurements using antibody arrays, although the most commonly used platform or detection method still remains to be determined. As the potential uses of antibody arrays are so varied, researchers or companies might find it necessary to become very adept at rapid assay development and customization, to meet specific and changing biological questions. The versatility of arrays, and the ability to test multiple reagents in parallel, should support those efforts.

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