

Antibody Arrays in Cancer Research

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Antibody arrays have valuable applications in cancer research. Many different antibody array technologies have been developed, each with particular advantages, disadvantages, and optimal applications. The methods have been demonstrated on various sample types, such as serum, plasma, and other bodily fluids; cell culture supernatants; tissue culture lysates; and resected tumor specimens. The applications to cancer research have included profiling proteins to identify candidate biomarkers, characterizing signaling pathways, and the measurement of changes in modification or expression level of cancer-related proteins. Further innovations in the methods and experimental strategies are broadening the scope of the applications and the type of information that can be gathered. These alternate formats and uses of antibody arrays include arrays to measure whole cells, arrays to measure enzyme activities, reverse phase arrays, and bead-based arrays. This article reviews the various types of antibody array methods and their applications to cancer research. *Molecular & Cellular Proteomics* 4:377–383, 2005.

Antibody microarrays enable the parallel detection of multiple proteins in low sample volumes. Continued improvements in the technology have increased the usefulness of the methods for basic and applied biological research, including cancer research. Several different versions of antibody microarray formats and methods have been developed, each with various advantages, disadvantages, and optimal applications. This review looks at the applications of those various technologies to cancer research.

The experimental features of microarrays have advantages for cancer research. The low sample volumes result in the consumption of small amounts of both precious clinical samples and expensive antibodies. The assays can be run efficiently in parallel, enabling studies on the large populations of samples that are necessary for marker discovery and validation. In addition, the assays can have good reproducibility, high sensitivity, and quantitative accuracy over large concentration ranges (1). Antibody and protein arrays are complementary and in some aspects preferable to separation-based and mass spectrometry-based technologies. Reproducibility and throughput can be higher, and the identities of the measured proteins are known or can be readily characterized.

Therefore, specific hypotheses regarding the nature of molecular alterations can be tested and generated, and the observed measurements can be biologically interpreted.

A common application of antibody arrays in cancer research is the identification of biomarkers or molecules that are potentially valuable for diagnosis or prognosis or as surrogate markers of drug response. The multiplex capability of antibody arrays allows the efficient screening of many marker candidates to reveal associations between proteins and disease states or experimental conditions. Multiplexed measurements also allow the evaluation of the use of multiple markers in combination. The use of combinations of proteins for disease diagnostics may produce fewer false positive and false negative results as compared with tests based on single proteins. Antibody microarrays, by increasing the number of proteins that can be conveniently measured in clinical samples, could more significantly take advantage of the benefit of using combined markers in diagnostics. Other example applications of antibody microarrays in cancer research are to evaluate the coordinated changes of members of signaling pathways or to measure changes in expression levels of a class of proteins, such as angiogenesis factors.

This review covers the major applications of antibody array technologies to cancer research. The first section gives an overview of the main features of the various antibody microarray technologies. Details of the technologies will not be covered extensively; reviews with a greater technological focus are available elsewhere (2–6). The next two sections cover applications to cancer research and are divided according to the type of sample analyzed: fluids (second section) and cells (third section). The final section looks at some of the related but alternate experimental antibody array strategies applied to cancer research.

TECHNOLOGICAL OVERVIEW

Many variants on antibody microarray technology have appeared. Fig. 1 presents the main experimental formats covered in this article. Two main types of formats are “label-based” assays (Fig. 1A) and “sandwich” assays (Fig. 1B). The methods are complementary and have respective advantages and disadvantages. In the label-based assay, the targeted proteins are labeled with a tag that allows detection after capture by an immobilized antibody. The *left drawing* of Fig. 1A shows “direct labeling” in which proteins are labeled with a fluorophore such as Cy3 or Cy5, and the *right drawing* shows “indirect detection” in which proteins are labeled with a tag that is subsequently detected by a labeled antibody. Label-based assays allow the co-incubation of two different

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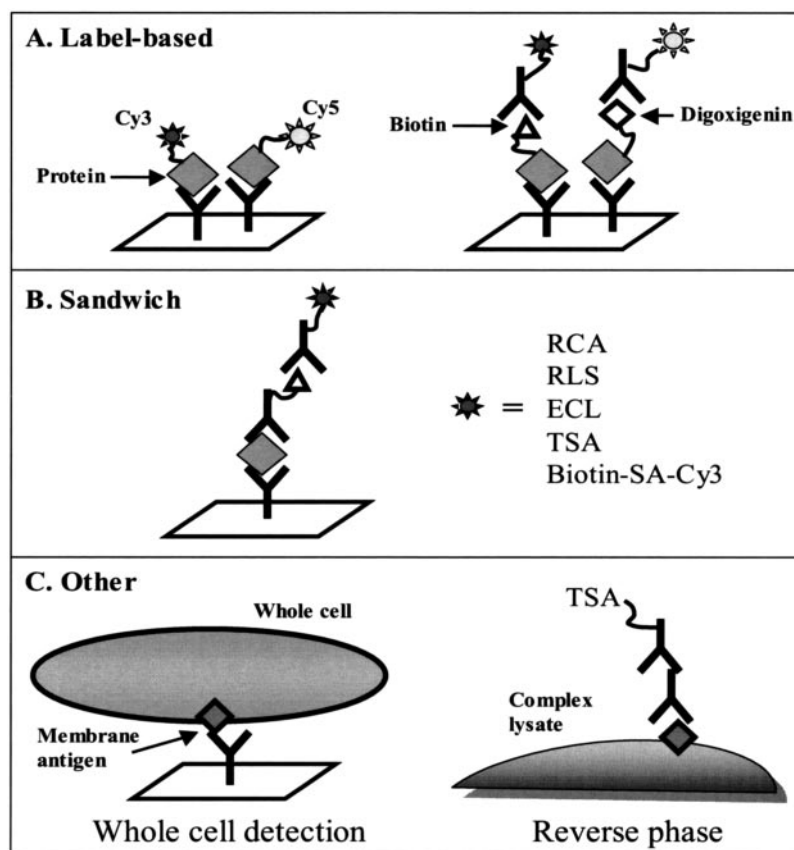


FIG. 1. **Antibody microarray assay formats.** *A*, label-based assays showing detection in two colors. The *left drawing* shows a direct labeling assay in which two protein pools are respectively labeled with Cy3 or Cy5. The *right drawing* shows indirect detection in which two protein pools are respectively labeled with tags such as biotin and digoxigenin. Labeled secondary antibodies detect the tags. *B*, sandwich assay. A matched pair of antibodies bind the unlabeled analyte followed by detection by a secondary antibody. Detection is through one of the methods listed at *right*: RCA, resonance light scattering (RLS), enhanced chemiluminescence (ECL), tyramide signal amplification (TSA), and streptavidin (SA). *C*, other strategies. The *left drawing* shows whole cell detection. Suspensions of cells are incubated on microarrays of antibodies targeting cell surface antigens. Cells bind to the array according to their expression of the targeted antigens, and the level of binding to each antibody spot is quantified by dark field microscopy. The *right drawing* shows reverse phase detection. Complex cell lysates are spotted onto surfaces, and the presence of particular proteins in the lysates are quantified by incubation of antibodies targeting those proteins. High sensitivity detection of the bound antibodies is possible through tyramide signal amplification (TSA).

samples, each labeled with a different tag, on the arrays. A benefit of that feature is that a reference sample may be co-incubated with a test sample to normalize for variation between spots in capture antibody concentration. Another benefit is that the assay is competitive: the analytes in the test and reference solutions compete for binding at the antibodies. Competitive assays have some advantages over non-competitive assays in linearity of response and dynamic range (7). Potential disadvantages of label-based detection are limited specificity and sensitivity and the possible disruption of the analyte-antigen interaction by the label.

Another format is the sandwich assay in which immobilized antibodies capture unlabeled proteins, and the captured proteins are detected by another antibody (Fig. 1B). The signals for detection may be generated by a variety of methods as listed on the *right* in Fig. 1B. Sandwich assays can have higher specificity than label-based assays since two antibodies tar-

get each analyte instead of just one. Detection sensitivity also can be higher due to reduced background. The assays are non-competitive since only one sample may be incubated on each array. Non-competitive assays result in sigmoidal binding responses with respect to analyte concentration (not linear as in the competitive assay) so that a “standard curve” of known concentrations of analytes is required to achieve accurate calibration of concentrations. Sandwich assays are more difficult to develop in a multiplexed setting relative to label-based assays. Matched pairs of antibodies and purified antigens may not be as readily available for each target, and the possibility of cross-reactivity between detection antibodies increases with additional analytes. For these reasons, multiplexed sandwich assays are considered to have a practical size limitation of 30–50 different targets. Arrays using label-based detection, on the other hand, are only limited by the availability of antibodies and the available space on a

substrate. Commercially available antibody arrays using label-based detection contain hundreds of antibodies (see Clontech and Schleicher & Schuell). Most of the applications described below make use of the methods shown in Fig. 1, A and B. Alternate strategies, such as those shown in Fig. 1C, are discussed later.

STUDIES ON BODILY FLUIDS OR SECRETED PROTEINS

Studies on proteins from fluids or cell culture secretions are technically easier than cell-based studies since all the proteins are soluble, and the samples require little preparative work. Applications include biomarker discovery and the profiling of cytokines to give information about signaling between cells. Blood serum or plasma is a particularly valuable sample source for cancer diagnostic research because of its rich biological information and because it can be used for minimally invasive, routine, and inexpensive screening. Several groups have reported on the use of antibody arrays to profile proteins present in human blood or other fluids. A recent study showed the application of antibody microarrays to the study of multiple proteins in the sera of prostate cancer patients and controls (8). The study used a two-color comparative fluorescence assay developed previously (1), similar to that depicted in Fig. 1A. The proteins serum samples from 33 prostate cancer patients and 20 controls were covalently labeled with either of the fluorescent dyes Cy3 or Cy5, and each sample was mixed with a reference protein solution (a pool of equal volumes of each serum sample) that was labeled with the different color fluorescent dye. Each sample-reference mixture was incubated on the surface of an antibody microarray containing 184 different antibodies targeting a variety of intracellular and secreted proteins, and the comparison of sample-specific fluorescence to reference-specific fluorescence at each antibody spot provided a measure of relative protein binding between the serum sample and the reference. The accuracy of the microarray measurements was evaluated by comparison with data from ELISAs, and the reproducibility of replicate data provided an effective means to filter out unreliable measurements. Five of the serum proteins had levels that statistically discriminated the cancer samples from the controls. This work established the feasibility of accurately and reproducibly measuring multiple proteins in serum samples using antibody microarrays.

An extension of the above work increased the sensitivity of the two-color comparative fluorescence assay by amplifying the fluorescence signal in both color channels (9). Rolling circle amplification (RCA)¹ was used to measure the relative levels of proteins from two serum samples, respectively labeled with biotin and digoxigenin, that had been captured on antibody microarrays. This method, called two-color RCA, enabled the detection of lower abundance proteins as com-

pared with the method described above. The accuracy and reproducibility of two-color RCA was verified, and the method was applied to the profiling of dozens of different proteins in sera from 24 cancer patients and controls. This method may be useful for the efficient and broad profiling of a wide variety of proteins in biomarker discovery applications.

The above studies used a label-based strategy (Fig. 1A). Sandwich assays on microarrays (Fig. 1B) also have been used by many groups to measure protein abundances in cancer fluids. Various detection methods have been used: rolling circle amplification (10, 11), resonance light scattering (12), enhanced chemiluminescence (13), tyramide signal amplification (14), and fluorescence (15, 16). Huang *et al.* (13) used arrayed sandwich assays to detect 24 different cytokines in conditioned media and serum samples. The antibodies were spotted onto membranes, and after incubation of samples on the membranes, bound antigens were detected by a mixture of biotinylated detection antibodies. The detection antibodies were quantified by enhanced chemiluminescence. The authors showed alterations in cytokine secretion from human glioblastoma cells upon treatment with TNF α . A similar antibody microarray using enhanced chemiluminescence detection of five different angiogenic factors found the levels of these factors 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). The company Molecular Staging produced another notable demonstration of multiplexed sandwich assays (10). Fifty-one different anti-cytokine antibodies were spotted in quadruplicate onto derivatized glass microscope slides. The authors used rolling circle amplification to amplify the signal from bound detection antibodies, resulting in detection limits in the low picogram per milliliter range for many antigens. Profiling of cytokine secretion from human dendritic cells induced by lipopolysaccharide or TNF α revealed both expected and novel secretions. Also interesting differences between lipopolysaccharide- and TNF-induced secretions were observed. This demonstration of highly quantitative, sensitive, and multiplexed cytokine analysis clearly showed the power and potential of the antibody microarray format.

Other groups reported the development of cytokine-targeting arrays for similar applications. Li *et al.* (16) described the development and optimization of antibody microarrays measuring cytokines. The authors compared the performance of multiple surfaces, buffers for the antibodies, blocking buffers, fluorescent labels, and antibody concentrations. A microarray-based sandwich assay using tyramide signal amplification to detect hepatocyte growth factor was used to identify elevated levels of hepatocyte growth factor in the sera of breast cancer patients (14). A novel application of antibody microarrays was the study of cytokines in tumor interstitial fluid, which perfuses the tumor microenvironment. Tumor intersti-

¹ The abbreviations used are: RCA, rolling circle amplification; TNF, tumor necrosis factor.

tial fluid samples were collected from breast tumor tissue and normal surrounding breast tissue and analyzed by mass spectrometry, Western immunoblotting, and cytokine-specific antibody microarrays (19). The microarrays were similar to those developed by Huang *et al.* (13), described above, and targeted 70 different cytokines. The characterization of cytokine levels in the fluid will be used with the other types of proteomic data to elucidate the interactions between the cells in the tumor environment.

TISSUE AND CELL CULTURE STUDIES

In addition to measuring protein abundances in fluids such as serum, cell culture supernatant, and interstitial fluid, antibody microarrays have been applied to the study of protein levels in cells from resected tissue and cell culture. Studies on cellular proteins can be more difficult than studies on fluids since the samples can be more complex and produce higher backgrounds or more interfering factors. Nevertheless multiplexed studies on intracellular proteins could be very important for a variety of applications, such as the identification of proteins with altered expression levels in tumors or the characterization of alterations to phosphorylation states and signaling pathways.

Antibody microarrays have been used to analyze proteins from frozen resected tumor tissue (20). Laser capture microdissection was used to remove selected regions of tumor and stroma from a frozen tissue sample. The isolated proteins were labeled with biotin, applied to antibody microarrays on nitrocellulose, and measured with chemiluminescence. The antibody microarray measurements were validated by Western blot and immunohistochemistry. The authors identified proteins both in the tumor tissue and in the surrounding stroma that had levels correlating with advancement of disease. This work demonstrated the important application of multiplexed protein measurements from surgically removed tissue specimens.

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 two-color comparative fluorescence (using Cy3 and Cy5 labeling) on microarrays containing 83 different antibodies. Thirty-two of the proteins had differential expression between the tumor and normal groups, and the levels of many of the proteins were confirmed by Western immunoblot. Two of the proteins, cyclin D1 and suppressor of cytokine signaling 1, showed an association with tumor prognosis. A similar study used antibody microarrays and two-color comparative fluorescence to compare protein levels between malignant and normal breast tissue from the same patient (22). A commercially available microarray from Clontech targeted 378 different proteins. Several were found to have higher levels in the malignant tissue, and the levels were confirmed by immunohistochemistry. Using larger sample sets, the approach could give some mechanistic insight into breast malignancy.

Cell culture studies also are useful in cancer research since they allow more in depth studies of particular properties of cells. The cytokine arrays developed by Huang *et al.* (13), as described above, were applied to the measurement of 35 different cytokines in lysates from cultured breast cancer cells and endometriosis tissue (23). Another study used two-color comparative fluorescence to examine the effect of UV irradiation on protein expression in apoptosis signaling pathways (24). Proteins were isolated from colon carcinoma cells grown in culture either before or after exposure to UV irradiation. The two pools were respectively labeled with either Cy3 or Cy5 and incubated together on microarrays containing 146 different antibodies spotted about 10 times each on coated glass slides. The authors identified multiple proteins that were either down-regulated or up-regulated in the UV irradiation-treated sample, and the changes in protein levels were confirmed by Western blot. Most of the up-regulated proteins in the irradiated cells were apoptosis regulators, consistent with the observation of induced apoptosis in those cells. This study showed that high quality antibody microarray data with biologically relevant implications could be derived using proteins from cell culture lysates. This type of experiment may be very useful in studying signaling pathways, particularly if accurate measurements of both phosphorylated and unphosphorylated forms of proteins can be made as described below.

Developments that allow the measurements of phosphorylation states of captured proteins promise to be very valuable for studying signaling pathways in relation to cancer. Nielsen *et al.* (25) are developing microarrays to study signaling in networks of interacting proteins. Sandwich assays were developed to measure both the abundances and phosphorylation states of ErbB2, epidermal growth factor receptor, and the transferrin receptor. Proteins are captured by the immobilized antibodies and are probed with antibodies targeting either the proteins or phosphotyrosine. By looking at the ratio of phosphotyrosine to total protein, the level of activation of the receptors may be measured. 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 (26). Cell extracts were incubated on arrays of antibodies targeting various cellular components of signaling pathways, and the bound proteins were probed with labeled anti-phosphotyrosine antibodies. Changes in the phosphorylation as a function of drug and growth factor treatment were measured on the microarrays and validated by mass spectrometric analyses. A related approach was taken to measure protein modifications using antibody arrays (27). Whole cell extracts were immunoprecipitated using antibodies targeting phosphoty-

rosine, 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.

OTHER EXPERIMENTAL STRATEGIES

This section covers some alternate experimental strategies for measuring the targets of multiple antibodies, particularly as applied to cancer research. These strategies are complementary to those described above and are potentially very valuable for cancer research. A novel application of antibody microarrays was the detection of the levels of cell surface proteins on whole cells without protein isolation (28). Suspensions of cells were incubated on antibody microarrays, and the amount of cells that bound to each antibody was quantified by dark field microscopy (Fig. 1C, *left*). 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 the phenotype of leukemia patients. Indeed they identified antigens that accurately discriminated between lymphocytes from chronic lymphocytic leukemia patients and normal lymphocytes. This method could be valuable for characterizing multiple membrane proteins in cell populations from other diseases. Further technical developments have better enabled such studies as demonstrated by the identification of drug-induced changes in cell surface antigens (29).

Another novel use of antibody microarrays was to profile enzyme activities in complex proteomes (30). The measurement of enzyme activity, rather than just abundance, is important for determining the functional state of certain proteins and may be valuable in cancer research. Previous research used gel-based strategies to determine the amount of labeled enzyme-specific probes bound to the active sites of particular classes of enzymes. To improve the sensitivity and specificity of the methods and to reduce sample consumption, a microarray platform for activity-based protein profiling was developed. Complex protein samples are treated with fluorescent activity-based probes, and the labeled enzymes are captured and detected on antibody microarrays targeting those enzymes. The activities of the enzymes 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. This novel application enhances the type of information that can be derived from antibody microarrays.

A complementary approach to antibody microarrays described earlier (Fig. 1, *A and B*) is the “reverse phase” microarray (Fig. 1C, *right*) in which multiple complex protein mixtures are spotted onto membranes and probed with antibodies targeting specific proteins. This method has the advantages that many samples can be analyzed in a single experiment and that non-soluble proteins are easily analyzed.

Potential limitations with the method are nonspecific binding of the antibodies to other components of the complex mixtures and low throughput in measuring many different antibodies. The reverse phase microarray was used to analyze proteins collected from resected prostate tumors, identifying changes in apoptosis pathways correlating with the progression of cancer cells (31). The method also was used to profile the binding of 52 different antibodies to lysates from 60 cell lines (the NCI-60) that represent a wide variety of cancers and that have been used for molecular profiling and drug screening studies (32). The data were correlated with expression profiling data to determine the relationship between protein levels and transcript levels. It was found that cell structure-related proteins showed a good correlation between mRNA and protein levels, but non-cell structure-related proteins did not. Reverse phase arrays also have been used to analyze components of signaling pathways in prostate cancer (33) and ovarian cancer (34) specimens that had been isolated by laser capture microdissection. Phosphospecific antibodies allowed the assessment of the activation states of some of the signaling proteins and the correlation of those states with clinical and pathological information (34).

Bead-based arrays are a potentially powerful complement to planar arrays (35). The Luminex bead array system is finding increased use in protein profiling applications. The system uses multiple, different fluorescent beads, each spectrally resolvable from the other and each coated with a different capture antibody. The beads are incubated with a sample to allow protein binding to the capture antibodies, and the mixture is incubated with a mixture of detection antibodies, each corresponding to one of the capture antibodies. The detection antibodies are tagged to allow fluorescent detection. The beads are passed through a flow cytometer, and each bead is probed by two lasers: one to read the color, or identity, of the bead and another to read the amount of detection antibody on the bead. Kits are available from various vendors (*e.g.* Rules-Based Medicine) that read up to 22 different analytes in a single assay. The method was used to monitor the secretion of several angiogenic factors, $\text{TNF}\alpha$, basic fibroblast growth factor, vascular endothelial growth factor, and transforming growth factor β , from cultured cells after exposure to chemotherapeutic agents (36). Only malignant cells secreted vascular endothelial growth factor, and the levels decreased with the addition of certain anticancer agents. Stromal cells in addition to malignant cells secreted basic fibroblast growth factor and transforming growth factor- β , which may contribute to tumor progression when secreted in the tumor environment.

SUMMARY AND OUTLOOK

The above reports show that the applications of antibody array methods to cancer research are increasing in scope and effectiveness. The various methods presented here are complementary with each other and with other proteomic meth-

ods, and they may be used together for added benefit as demonstrated in a study of proteins in breast cancer cells using cytokine arrays, reverse phase arrays, and bead-based arrays in conjunction with two-dimensional gels (37). These and similar approaches are certain to be valuable in a broad range of research fields. For example, a study on the inflammatory response in osteoarthritis using antibody microarrays revealed cytokines secreted by human chondrocytes in response to interleukin-1 and TNF (38), and another report showed the development of a tool to monitor biomarkers of rheumatoid arthritis (39). In another application to immunology, reverse phase arrays were used to study signaling in T-cells and stimulated phosphorylation in rare subpopulations of T-cells (40).

As the diversity of proteins is so much greater than nucleic acids, true "proteomic" antibody arrays, targeting a significant portion of a tissue proteome, will not be practical in contrast to "genomic" level DNA arrays. The arrays will likely be developed for specific hypotheses in specific tissue types, such as the plasma proteome. However, high throughput antibody production and screening methods could enable the efficient probing of a large number of novel proteins in an "affinity proteomic" approach (41, 42). Some have proposed phage display and related methods as tools to rapidly produce antibodies for broad screening applications (43, 44). Regardless of the antibody production methods, antibody specificity and performance must be validated, and as that process can be lengthy, antibody microarrays with highly validated specificities will likely target select sets of proteins for specific applications. Undoubtedly additional novel variations and innovative uses of the methods will continue to appear and produce new opportunities for discovery. As these methods continue to mature and are used by the wider research community, measurable benefits to cancer patients may be forthcoming.

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