Review

Protein microarray detection strategies: focus on direct detection technologies

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Accepted 8 April 2004
Available online 28 May 2004

Abstract

Protein microarrays are being utilized for functional proteomic analysis, providing information not obtainable by gene arrays. Microarray technology is applicable for studying protein–protein, protein–ligand, kinase activity and posttranslational modifications of proteins. A precise and sensitive protein microarray, the direct detection or reverse-phase microarray, has been applied to ongoing clinical trials at the National Cancer Institute for studying phosphorylation events in EGF-receptor-mediated cell signaling pathways. The variety of microarray applications allows for multiple, creative microarray designs and detection strategies. Herein, we discuss detection strategies and challenges for protein microarray technology, focusing on direct detection of protein microarrays.

Published by Elsevier B.V.

Keywords: Protein microarray; Catalyzed reporter deposition; Molecular profiling; Fluorescence; Chromogens

1. Introduction

A number of interesting factors have propelled protein microarrays into the limelight. First, miniaturization of assay methodologies has fueled the growth of microarray technology. Ekins et al. have provided evidence that, within limits, miniaturization of spot size can increase sensitivity of detection (Ekins, 1989; Ekins et al., 1990; Templin et al., 2002). In parallel, there has been a growing excitement to move beyond the genome to the proteome. Finally, the success of DNA-based array technology has encouraged the transition to protein microarrays by providing platforms and software tools (Ge, 2000; Liotta and Petricoin, 2000; MacBeath and Schreiber, 2000; Knezevic et al., 2001; Paweletz et al., 2001; Zhu and Snyder, 2001, 2003; Charboneau et al., 2002; Lal et al., 2002; Lesaicherre et al., 2002; MacBeath, 2002; Cutler, 2003; Miller et al., 2003; Wilson and Nock, 2003).

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Gygi et al. (1999) and Anderson and Seilhamer (1997) have demonstrated the lack of correlation between gene transcripts and protein abundance. Protein microarrays provide information not obtainable by gene arrays. This is not unexpected because gene arrays cannot provide information about protein posttranslational modifications or protein–protein interactions. Protein microarrays, on the other hand, can be designed to monitor these molecular interactions. Proteins are the action molecules, or verbs of the cell, directing inter and intracellular interactions. Microarrays provide a means of differential display of a cell population’s proteome as well as high-throughput formats for drug discovery (Greenbaum et al., 2002; Ring and Ellis, 2002; Wilson and Nock, 2003). This differential display can be used for development of cellular circuit maps, revealing the network of upregulated and downregulated proteins in disease and non-disease cell populations (Bowden et al., 1999; Hunter, 2000; Jeong et al., 2000; Blume-Jensen and Hunter, 2001; Celis and Gromov, 2003). Microarray applications include protein–protein, DNA–protein, RNA–protein and protein–ligand interactions. Comparison of protein profiles for multiple patient samples or the same patient at multiple time points during a treatment regimen is a current application of protein microarray technology related to drug discovery.

Cellular proteomic changes generated by defective genetic transcripts leads to disease by altering a cell’s response to its microenvironment (Liotta and Kohn, 2001). This knowledge coupled with the information generated by gene and protein microarrays is providing a basis for molecular profiling of diseased cell populations. By combining the information gleaned from genomics and proteomics, paired with the strength of bioinformatic data analysis tools, we foresee future technology capable of mapping the proteomic network of diseased cell populations (Liotta et al., 2001; Petricoin et al., 2002). Protein microarrays are one of the technologies that will help us realize this vision.

2. Protein microarray formats

Microarrays are miniaturized bait-and-capture assays (Liotta et al., 2003). A variety of applications and formats have been devised, such as antibody microarrays, protein microarrays and tissue microarrays. These various immobilization and detection strategies have been devised depending on which molecules are used as the bait molecules and which are used as the capture molecules. The common element for each type of microarray is the immobilization of bait molecules on a substratum, either as a homogeneous or heterogeneous spot (Liotta and Petricoin, 2000; MacBeath, 2002; Zhu and Snyder, 2003). Bait molecules may be aptamers (Jensen et al., 1995; Petach and Gold, 2002), antibodies (Sreekumar et al., 2001; Lal et al., 2002; Templin et al., 2002; Wilson and Nock, 2003), cell lysates (Paweletz et al., 2001), phage or recombinant protein/peptide (MacBeath and Schreiber, 2000; Kukar et al., 2002; Fall et al., 2003; Zhu and Snyder, 2003), a nucleic acid (Ge, 2000; Petach and Gold, 2002; Schaeferling et al., 2002; Weng et al., 2002; Xu et al., 2002), or a tissue (Kononen et al., 1998). The capture molecule may be a complex biologic mixture, such as serum or a cell lysate, an antibody or ligand. Enzyme-linked immunosorbant assay (ELISA)-style sandwich assays, employing an immobilized antibody, a capture protein and a second, labeled antibody have been adapted to the microarray format (Joos et al., 2000; Delehanty and Ligler, 2002; Pawlak et al., 2002). Herein, we focus on detection strategies for direct detection protein microarrays and the accompanying challenges for future technologies.

3. Protein microarray challenges

Protein microarray technology is not as straightforward as DNA-based microarrays owing to the complex structure of proteins. As yet, PCR amplification does not exist for proteins, necessitating high-throughput technologies such as microarrays for extracting information from the proteome. Proteins are comprised of 20 amino acids capable of forming complex tertiary and quaternary structures. Proteins may be hydrophobic, hydrophilic, acidic or basic. Posttranslational protein modifications, such as glycosylation and phosphorylation, complicate attempts at determining protein interactions based solely on amino acid sequence. Protein immobilization to a substratum occurs via multiple, poorly understood
interactions consisting of van der Waals, electrostatic and hydrophobic interactions. DNA, on the other hand, composed of only four nucleotides, is a molecule with a defined hydrophilic backbone and structure. The negatively charged DNA molecule binds a substratum in a defined manner. The complimentary structure of DNA facilitates querying with detection probes. This molecular variability, coupled with the wide dynamic range of protein concentrations found in any sample, presents challenges for protein microarray immobilization and detection strategies that are not as prevalent as for DNA microarrays (Lal et al., 2002; Celis and Gromov, 2003; Zhu and Snyder, 2003).

Protein detection strategies must address the challenges posed by the complex nature of a proteome. The ability to detect low abundant proteins in a complex biological mixture is the first hurdle. Amplification techniques (Bobrow et al., 1989, 1991; Hunyady et al., 1996; King et al., 1997) with stringent amplification chemistries have been developed for chromogenic detection (Paweletz et al., 2001; Charboneau, 2002) and fluorometric detection (Larison et al., 1995; Berggren et al., 1999; Panchuk-V oloshina et al., 1999; Zhu et al., 2001; Bacarese-Hamilton et al., 2002; Kukar et al., 2002; Wiese, 2003) of proteins.

The second hurdle faced by protein microarray detection strategies is the requirement for specific high-affinity antibodies and ligands as probe molecules. Antibodies cannot be manufactured with known affinity and specificity. This requires individual validation of antibody specificity and sensitivity prior to use as a probe for protein microarrays (Templin et al., 2002; Liotta et al., 2003). Fortunately, antibody validation can be accomplished with Western blotting, using similar sample material as the protein microarray. A single band on Western blot, at the specified molecular weight, serves as an indication of specificity and sensitivity for the proposed antibody. In addition, positive and negative controls, such as recombinant peptides, can be printed on each protein microarray providing a means of quality control for printing and detection methods.

The antibody affinity also determines the linearity range of the assay. Linearity can only be achieved when the concentration of the analyte and antibody are matched to the affinity constant. Multiplexed formats containing multiple antibodies with varying affinities will not be able to achieve linearity for all analytes in each spot (Liotta et al., 2003).

Sandwich assays (Templin et al., 2002; Zhu and Snyder, 2003) require two distinct antibodies that are able to bind two available, non-overlapping epitopes on the same protein. This requirement doubles the stringency placed on the detection system due to the two different affinity constants for each tagging reaction (Liotta et al., 2003).

The third hurdle affecting successful detection of microarrays is the denatured vs. native state of proteins. Foremost, completely different lysis conditions, buffers, antibodies and reagents would be required for the detection of native vs. denatured proteins. Secondly, numerous antibodies are made against peptides rather than native proteins. In the process, the antigen is denatured allowing the epitope to be linearized. These antibodies may not bind to proteins in the native state, limiting the ability to detect protein–protein interactions.

3.1. Tissue microarrays

Tissue microarray (TMA) technology was developed as a high-throughput screening tool for DNA, RNA and protein in large numbers of specimens (Kononen et al., 1998). A discussion of tissue microarrays is warranted to avoid terminology confusion with protein microarrays. Additionally, tissue microarray analysis complements proteomic disease investigation.

As many as 1000, 0.6-mm cylindrical tissue biopsies from paraffin-embedded or frozen tissues can be assembled into a single TMA (Kononen et al., 1998; Hoos and Cordon-Cardo, 2001; Hoos et al., 2001; Schoenberg Fejzo and Slamon, 2001). Hundreds of 4–8-μm sections can be cut from each array and analyzed in parallel for a large number molecular markers allowing for high-throughput correlative analysis (Kononen et al., 1998). TMAs represent a valuable tool in cancer research for validation studies, speeding the translation of potential biomarkers and therapeutic targets into clinical applications. TMA construction can also be tailored to more specific needs. Multi-tissue or multi-tumor arrays composed of multiple tissue or tumor types can be assembled for analysis of molecular alterations across varied sam-
Specific TMAs can be constructed to study molecular changes during neoplastic progression for a specific tumor type or to determine the prognostic value of a biomarker in tissues where clinical data are available. While TMA technology has been used most widely in cancer research, its applications can obviously be extended to other fields of disease research (Simon and Sauter, 2002).

TMAs are also potentially useful for the analysis of endpoints in large-scale or multicenter studies, as well as for the testing, optimization and standardization of antibodies and immunohistochemical techniques in intra- and interlaboratory settings (Moch et al., 2001; Hsu et al., 2002).

While TMAs can be used for genomic applications, a significant proportion of clinicopathological evaluation of tissue involves immunohistochemical staining of tissue sections for biomarkers used in diagnostic, prognostic and treatment considerations. A number of studies have demonstrated the utility of TMAs for these purposes (Bowen et al., 2000; Dolled-Filhart et al., 2003; Fan et al., 2003). As with any emerging technology, there are issues that must be addressed. One major concern surrounding TMA technology is that the small size of the tissue sample is not representative of the whole tumor. Evaluation of Her2/neu expression in TMAs of breast tumors demonstrated that two core tissue samples per tumor resulted in 95% concordance with whole tumor data suggesting that twofold redundancy in tissue representation on an array is sufficient for many analyses (Camp et al., 2000). However, analysis of the retinoblastoma protein expression phenotype in fibroblastic tumors by TMA suggested that three or more tissue cores may be necessary to assess more complex protein expression patterns, and TMA technology may not be the appropriate option for all of these complex phenotypes (Hoos et al., 2001). A larger issue for TMA technology is tissue heterogeneity. Tissue is inherently comprised of multiple cell types, all of which may have some cellular proteins in common. When TMA technology is applied to the investigation of phosphoprotein detection, almost every cell in any given field can have the same activated protein, i.e., ERK is activated in infiltrating lymphocytes, epithelium and stroma.

As with any immunohistochemical procedure, it is important to include positive staining controls among the TMA samples to control for antigen retrieval and other antibody-related problems. Because formalin-fixed, paraffin-embedded tissues can present limitations for the analysis of RNA and some proteins, some investigations may require construction of frozen tissue microarrays. Frozen TMAs eliminate the need for antigen retrieval compared with paraffin-embedded tissues, but sacrifice quality in histology and require larger diameter core samples than paraffin-embedded core samples to maintain array integrity (Hoos and Cordon-Cardo, 2001; Schoenberg Fejzo and Slamon, 2001). Traditionally, immunohistochemical staining of tissues is described by a manual, categorical scoring system. Such subjective methods of analysis can lead to bias and reproducibility problems in sample evaluation.

The advantage TMA technology provides in terms of multiplexed, parallel, high-throughput processing of samples underscores the need for more quantitative, automated methods for data analysis. Development of detection strategies that allow for signal quantification on a continuous numerical scale as opposed to the current categorical methods could reveal subtle but biologically relevant differences that are lost in current manual methods. Recent analyses are combining automated, quantitative fluorescence techniques with TMA technology to fill this need (Camp et al., 2002, 2003; Rao et al., 2002). One study used a quantitative fluorescence image analysis method with TMAs to show distinctive expression patterns of BRCA1 protein in high- and low-grade ovarian cancers, adjacent dysplastic tissue, and distant non-tumor tissue that could not be discerned by traditional immunohistochemical staining method (Rao et al., 2002). An alternative method, termed Automated Quantitative Analysis (AQUA), incorporates algorithms to analyze both subcellular localization and quantify protein expression in tissue microarray samples and compares favorably with standard pathological assessment (Camp et al., 2002). Use of this method to quantify HER2/neu expression in a TMA containing 300 breast cancers and 84 normal tissue samples revealed that normal tissue and approximately 18% of the breast tumors expressed similarly low but detectable levels of HER2 protein. This category was not definable by manual scoring of the same tissues. Interestingly, these low HER2-expressing tumors were as aggressive clinically as HER2-overexpressing tumors (Camp...
et al., 2003). TMA technology combined with automated, highly quantitative detection and measurement strategies can be a powerful screening tool for the presence of molecular markers at the tissue level.

4. Protein microarray detection strategy classifications

Current detection strategies are classified as (1) label-free methods and (2) labeled probe methods.

**Current Detection Strategies**

**Label-free**

A. Mass Spectrometry

![Mass Spectrometry Diagram](image)

B. Surface Plasmon Resonance (SPR)

![Surface Plasmon Resonance Diagram](image)

**Labeled-Probes**

C. Direct

- Mixture of immobilized proteins
- Specific detection with labeled antibody

D. Indirect

- Immobilized antibodies
- Probe with labeled proteins

E. Sandwich

- Immobilized antibody + protein
- Detection with labeled second antibody

Fig. 1. Current detection strategies for protein microarrays. Label-free detection methods such as mass spectrometry (panel A) and surface plasmon resonance (SPR) (panel B) imaging do not require labeling molecules that may affect protein activity. Currently, there are three distinct approaches for labeled probe detection. The label may be a chromogen, a fluorophore or a radioactive isotope. Direct detection strategies (panel C) employ a labeled antibody to directly bind to the target molecule immobilized on a substratum. Amplification strategies based on avidin–biotin binding enhance the sensitivity of detection. Indirect detection strategies (panel D) utilize an immobilized antibody for capturing labeled, specific molecules from a complex mixture of query molecules. Sandwich assays (panel E) require two distinct antibodies for detection of a capture molecule. The first antibody is immobilized on the substratum, the molecule of interest binds to its cognate antibody. A second, labeled antibody detects the bound antibody-capture molecule complex.
Label-free methods are mass spectrometry (Liotta and Petricoin, 2000; Liotta et al., 2001; Figeys, 2002; Batorfi et al., 2003), surface plasmon resonance imaging (SPR) (Johnsson et al., 1991), and atomic force microscopy (Jones et al., 1998). Mass spectrometry (Fig. 1A) microarrays utilize a protein-selective surface, such as hydrophobic, ionic, or biological surfaces for immobilization of a complex protein solution. Ions liberated from the surface by laser desorption/ionization fly to a detector and are classified based on their mass/charge ratio. Genetic algorithms and neural network data analysis are used for data mining and disease/non-disease data clustering analyses.

SPR detectors (Fig. 1B) are optical biosensors for monitoring biomolecular interactions. A bait molecule is immobilized on a thin metal film, typically gold or silver. Incident light is directed at a sharp angle to the side of the metal film opposite of the bait molecule. The light is reflected from the film at a certain angle. Changes to the bait molecule on the film, such as binding of a cognate antigen, causes a change in the electrons in the metal film, causing the angle of the reflected light to vary from the original angle. Measurement of the angle of reflectance indicates a binding event between the immobilized antibody and the capture molecule.

Atomic force microscopy applications for protein microarrays capitalize on the change in height of an immobilized antibody upon binding of its complimentary antigen (Binnig et al., 1986; Dammer et al., 1996; Allen et al., 1997). Label-free methods generally require sophisticated equipment not available in all laboratories or clinics.

4.1. Labeled probe methodology

Labeled probe detection methods (Fig. 1C–E) have evolved from clinical immunoassay protocols, incorporating fluorescence, chromogenic and radioactive labeling strategies for detection of immobilized targets. Labeled probe detection methods differ based on the immobilized bait molecule. The capture molecule itself may be detected directly as in antigen capture assays (Haab et al., 2001; Knezevic et al., 2001; Sreekumar et al., 2001; Fall et al., 2003). This format is referred to as a forward phase or antibody array (Fig. 1D). A variety of antibody bait molecules may be immobilized and probed with a labeled complex biologic mixture, allowing measurement of multiple analytes simultaneously for a given sample.

Sandwich assay formats (Fig. 1E) rely on immobilized antibody for capturing the protein of interest, while a second labeled antibody, directed against the captured protein, is used for detection (Delehanty and Ligler, 2002; Pawlak et al., 2002). Both antigen capture and sandwich assays require two distinct antibodies capable of binding separate epitopes on the protein of interest. The requirement for two distinct antibodies, each with its own affinity constants, severely constrains this array design.

Direct capture assays (Fig. 1C) consist of immobilized proteins on a substratum, and the protein of interest is detected using labeled antibodies (Graf and Friedl, 1999; Paweletz et al., 2001; Chen et al., 2003; Liotta et al., 2003). The direct capture microarray is also referred to as a reverse-phase protein microarray (RPA). Each array consists of a complex mixture of proteins in each spot, and each array is queried with a single probe (Liotta et al., 2003). An RPA may be comprised of hundreds of different patient samples, allowing comparison of a single analyte across multiple samples.

5. Detection strategies

Classical methods of protein analysis such as electrophoresis and liquid chromatography are generally time consuming, labor intensive and lack high-throughput capacity. The sensitivity limits for these methods is a function of the detection system, sample preparations and separation techniques (Weinberger et al., 2000). Sample preparation techniques introduce variables resulting in protein loss or degradation, making detection of low abundant proteins marginal at best. Miniaturization technology introduced for DNA microarray technology has been adapted for protein microarrays, leading to functional proteomic analysis. The detection methods developed for microarrays generally depend on the microarray format and substratum. Direct detection methods may capitalize on signal amplification techniques with a chromogenic or fluorescent probe that is compatible with the substratum. Indirect detection methods generally do not employ amplification techniques due to the direct
labeling of the probe, which is either the protein of interest or a second, labeled antibody.

5.1. Substratum selection

As mentioned above, there are numerous permutations of immobilization strategies and substrata for bait molecules. Current detection strategies have evolved from immunoassays, radioimmunoassays and ELISA assays (Weinberger et al., 2000). The selection of a substratum is the first consideration in selecting a detection method for reverse-phase protein arrays. The ideal substratum has common attributes that enhance the signal-to-noise ratio of the analyte: minimal autofluorescence, limited nonspecific binding, high surface area-to-volume ratio, inert to biological molecules and compatibility with available detection methods. Planar surfaces, common substrata for DNA microarrays, lack sufficient surface area per spot for binding biological sample constituents in the femtomolar range (Liotta et al., 2003). Microporous surfaces and membranes such as nitrocellulose, nylon and polyvinylidene difluoride (PVDF) have high surface area-to-volume ratios, high binding capacity and ease of use, making them amenable to protein microarrays. Proteins have been successfully immobilized on nitrocellulose (Graf and Friedl, 1999; Ge, 2000; Paweletz et al., 2001; Charboneau et al., 2002; Kukar et al., 2002; Tonkinson and Stillman, 2002), aldehyde-treated glass (Zhu et al., 2001), epoxy-derivatized slides (Chen et al., 2003), BSA-N-hydroxysuccinimide-treated slides (MacBeath and Schreiber, 2000), silanized glass slides (Lesaicherre et al., 2002) or nickel-coated slides (Zhu et al., 2001).

5.2. Chromogenic detection

Chromogens are molecules that serve as a substrate for an enzymatic reaction that generates a colored product. The most common microarray application of immunoenzymatic reactions is where an antibody is labeled with an enzyme. The enzyme acts as a colorless substrate generating a colored precipitate. ELISA reactions produce soluble products that diffuse through the substratum of a microarray, precluding detection of immobilized proteins. Chromogenic detection of protein microarrays produces permanent signals that are easily visualized for analysis. Commonly used enzymes for the chromogenic reactions are horseradish peroxidase (HRP) and alkaline phosphatase (AP). These enzymes act on a variety of colorless chemical substrates, each generating a different colored product. A word of caution when investigating chromogenes for use on microarrays: some of the precipitates are soluble in alcohol, requiring the absence of organic solvents in the test system after the reaction is complete. The most commonly used enzymes and substrates are listed in Table 1.

The signal intensity of chromogens varies according to the substrate. DAB is a commonly used chromogen with HRP and is applicable to microarray detection with femtomolar sensitivity (Paweletz et al., 2001; Charboneau et al., 2002). This level of sensitivity is necessary for applications to biological response monitoring and is necessary for human clinical trials. Protein microarray detection with DAB incorporates a signal amplification procedure based on catalyzed reporter deposition of substrate (Bobrow et al., 1989, 1991; Hunyady et al., 1996; King et al., 1997) using commercially available reagents (CSA, DakoCytomation, Carpinteria, CA). The microarray is blocked for endogenous peroxidase, avidin, biotin and protein activity prior to the addition of primary antibody. A biotinylated secondary antibody, directed against the primary antibody, is used as a starting point for signal amplification. A streptavidin–biotin complex (SABC) decorates the secondary antibody. Biotin-tyramide deposition in the area of the SABC acts as the amplification reagent. HRP bound to the tyramide cleaves the DAB, resulting in a brown precipitate with excellent signal-to-noise ratio. Following this method, Paweletz et al. (2001) were able to detect as few as 5000 molecules of PSA per microarray spot and also were able to profile the state of pro-survival proteins in normal prostate epithelium and prostate intraepithelial neoplasia with inter-slide and intra-slide coefficients of variation of 7% and 5%, respectively.

The DAB precipitate is stable and produces an intense signal with relatively low background. The signal may be further intensified with nickel, copper, silver, gold or cobalt to enhance the staining (Hsu and Soban, 1982). A disadvantage of DAB is the potential mutagenicity of the substrate and the need for proper disposal of chromogens. Staining reactions using the substrate TMB (Josephy et al., 1982), a non-carcinogenic, non-mutagenic derivative of benzidine, are
more sensitive than DAB reactions, although increased washing and blocking may be required due to higher background staining. Reactions using the substrates CN and ACE are less sensitive, but may be useful for double staining. CN products may diffuse from the site of precipitation while the AEC precipitate has a tendency to fade if exposed to light (Boenisch, 2001). The Fast Red TR/AS-MX substrate is commonly used for AP immunostaining. The staining may fade when dried but regains brightness upon rehydration. This limits its applicability to microarray detection strategies requiring scanning of a dried slide for image analysis. BCIP/NBT yields a reliable signal with low background but is less sensitive than Texas Red (Streit and Stern, 2001).

5.3. Chemiluminescent detection

Chemiluminescent detection methods for microarrays are based on Western blotting protocols for detection of antigen-bound antibodies with secondary antibodies conjugated to alkaline phosphatase or horseradish peroxidase. The enzymatic oxidation of a substrate, such as luminol, produces a prolonged emission of light, which is captured on X-ray film, a phosphor imager or with a CCD camera. Nonspecific protein-binding sites on the printed microarray are blocked with suitable blocking solutions, typically dilute bovine serum albumin or casein solutions (Paweletz et al., 2001; Charboneau et al., 2002; Fall et al., 2003). Chemiluminescent detection methods may be applied to any of the labeled probe detection methods (Fig. 1C–E). Amplification strategies such as the biotinyl–tyramide described above can be coupled to chemiluminescent detection (Paweletz et al., 2001). Fall et al. demonstrated the utility of chemiluminescent detection in a IgE allergen array. Purified and recombinant allergens were immobilized on slides coated with 1% (3-glycidyloxypropyl) trimethoxysilane (GOPS). A 25-µl sample of serum was used to screen 24 different allergens. Detection with streptavidin-HRP reagents produced results with an intraslide standard deviation of 2.6–7.6% and inter-slide standard deviation of 7.9%. Advantages of chemiluminescent detection methods are the ability to create a permanent record of results with film-based detection, high sensitivity and speed.

5.4. Fluorescence-based detection

Ekins et al. (1990) described fluorescence applications to microarrays in 1990. Since then, multiple
applications have been devised for direct detection of protein microarrays (Zhu et al., 2001; Kukar et al., 2002; Lesaicherre et al., 2002; Chen et al., 2003). Fluorescent molecules absorb photons of light energy from an external light source. This causes an excitation of electrons within the molecule and an emission of light at a different wavelength than the incident light. Fluorophores, like chromagens, exist in many formulations and have defined emission spectra. Fluorescein, rhodamine (Texas Red), phycobiliproteins, nitrobenzoxadiazole (NBD), acridines, cyanines and Bodipy compounds are commonly used for protein labeling. Selection of fluorophores for use with microarrays depends on sample type, substratum, emission characteristics and number of analytes to be assayed per array. Not all substrata are compatible with fluorescence detection strategies due to inherent autofluorescence of the material. Autofluorescence can cause significant reduction in signal-to-noise ratios. Nitrocellulose-coated slides cause light scatter and higher background as compared to aldehyde treated slides with laser scanner detection methods (Kukar et al., 2002), limiting the use of nitrocellulose substrata for fluorescent detection methods. In addition to autofluorescence of the substratum, the sample may have components that interfere with a selected fluorophore. Examples are flavins and flavoproteins found in liver and kidney tissue. Flavins autofluoresce and emit light in the same region as fluorescein, limiting fluorescein use in samples rich in flavoproteins (Pawley, 1995).

One needs to be mindful of photobleaching and quenching of fluorophores that may decrease the total signal observed on a microarray. The Cy3 and Cy5 dyes are commonly used for fluorescent detection for overcoming the effects of photobleaching and quenching. Cy3 and Cy5 are well suited to fluorescence detection strategies due to their decreased dye interactions, increased brightness and the ability to add charged groups to the molecules (Pawley, 1995).

Total protein determinations may be made directly on microarrays with a Sypro™ Ruby blot stain (Molecular Probes, Eugene, OR) (Berggren et al., 1999). A ruthenium organic complex interacts noncovalently with proteins immobilized on nitrocellulose (Fig. 1C). The stained proteins are excited with UV or visible light, with an emission spectrum of 618 nm. The dye is compatible with immunoblotting and mass spectrometry and is applicable to microarrays printed on nitrocellulose substrata. This type of total protein analysis is useful for minute or limited sample volumes in which a standard protein spectrophotometric analysis would not be feasible.

Fluorescently tagged proteins may be used for detection of immobilized molecules on a microarray as in an indirect labeled probe detection strategy or sandwich microarrays (Fig. 1D and E). Recombinant proteins with red and green fluorescent protein tags were used by Kukar et al. (2002) for investigation of protein–protein interactions. Green fluorescent protein antibody arrayed on nitrocellulose was probed with a GFP-RFP fusion protein. Image analysis showed colocalization of both proteins, allowing dual labeling of protein molecules. In addition, the fluorescent signals were proportional to the amount of arrayed protein, validating the use of fluorescent tags for protein microarray detection.

Chen et al. (2003) coupled fluorescent labels with enzyme inhibitors for enzyme class and specificity determinations. The inhibitors, fluorophosphonate, vinyl sulfone and phosphatase were bound to Cy3 molecules and used for probing immobilized enzymes. The inhibitor–dye complex binds covalently to the enzyme, making them useful for identification of class-specific enzymes and a high-throughput screening tool of potential enzyme inhibitors. Sensitivity of the assay was determined to be $10^{-21}$ mol enzyme (Chen et al., 2003).

Streptavidin–biotin amplification chemistries may be applied to fluorescence detection strategies. Zhu et al. (2001) arrayed GST-yeast fusion proteins on nickel-coated glass slides for investigation of protein–protein interactions. The microarrays were probed with biotinylated calmodulin with calcium. The biotinylated protein was detected with Cy3-labeled streptavidin. The microarray format allowed the identification of new calmodulin-interacting proteins (Zhu et al., 2001).

5.5. Radioactive decay-based detection

Detection of microarrays with radioactive isotopes is not widely used due to health and safety issues and long detection times (up to 10 h). However, the ability to incorporate $^{32}$P in protein, DNA and RNA enabled the production of a universal array for the detection of protein–protein, protein–DNA, protein–RNA and protein–ligand interactions (Ge, 2000). Purified pro-
Proteins immobilized on a nitrocellulose membrane were probed sequentially with a \(^{32}\text{P}\)-labeled protein probe, a \(^{32}\text{P}\)-labeled (ds)DNA probe, a \(^{32}\text{P}\)-labeled SV40 pre-mRNA probe, as well as a \(^{125}\text{I}\) triiodothyronine probe. A series of blocking and washing protocols were devised for optimization of each probe type. Signal detection was visualized by autoradiography and quantified with a densitometer. The advantages of this system are the ability to fully active proteins and to quantify the amount of protein or biomolecules on the array.

Prior to their success with fluorescently labeled probes, Zhu et al. (2000) analyzed the yeast proteome for in vitro kinase activity with \(^{32}\text{P}\)-g-ATP. Recombinant proteins were immobilized on microarrays made with the silicone-elastomer PDMS. Seventeen different kinase assays were prepared and quantified using high-resolution phosphorimaging. Their high-throughput system allowed comparison of the protein kinase functional relationships with one another (Zhu et al., 2000).

6. Conclusion

The direct capture or reverse-phase arrays described above are currently being employed to ongoing clinical research trials at the National Cancer Institute (NCI), Warren G. Magnuson Clinical Center. This type of protein microarray shows utility in deciphering cell signaling networks with the precision and sensitivity required for use with human tissue samples (Liotta et al., 2003). Changes in phosphorylation status or cleaved states of key signaling proteins are being evaluated via microarray for evaluation of this technology to human diagnostic and prognostic testing. The goals of the NCI clinical trials are (1) the evaluation and optimization of the microarray technology for specimen acquisition and processing and (2) the transition from basic research in combinatorial therapy to patient selection of targeted therapies.

Preliminary data from these clinical trials are revealing the importance of monitoring multiple cell signaling endpoints, for mapping an entire cellular network, rather than focusing on a defined biomarker or set of biomarkers. Cellular signaling networks are designed for redundancy and with a capacity to reorganize or rewire if one pathway becomes blocked, as with single agent chemotherapy. Identification of these rewired pathways will reveal relevant information for designing individual, targeted therapies and/or combinatorial therapies directed at multiple nodes in a cell signaling cascade. Immobilization of the entire cellular proteome, in the reverse-phase format, permits true multiplexing of phosphorylation specific endpoints, achieving the goal of cell signaling mapping. Multiple-patient samples or single-patient samples at multiple timepoints before, during and after therapy may be assayed under identical experimental conditions allowing comparison of patient samples across a microarray.

The availability of public software for protein microarray analysis enhances the high-throughput capabilities of the microarrays independently of the detection strategy. Direct capture format microarrays may be printed and detected in almost any laboratory using commercially available instruments and reagents. Currently, the detection strategies employed for direct capture microarrays rely on clinical immunoassay detection methods. While these systems achieve sensitivity in the femtomolar range, future technologies may allow more sensitive, multi-analyte analysis. The need for high-affinity, specific antibodies for detection is currently the major weakness of the protein microarray technology. Future applications of Quantum dot technology (Wu et al., 2003), immuno-fluorescent semiconductor material, may be applicable to subcellular multi-analyte analysis. Continued research in nanotechnology may be the key to optimizing the microarray detection strategies for patient-targeted therapy.

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