

Development of Functional Protein Microarrays for Drug Discovery: Progress and Challenges

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Abstract: Functional protein microarrays promise new approaches to address longstanding challenges in drug discovery and development, with applications ranging from target identification to clinical trial design. However, their widespread adoption will be contingent upon a robust ability to develop and manufacture arrays in support of these applications. This review will address the major areas of relevance to the development of functional protein microarrays; protein content, surface chemistry, manufacture and assay development. Successful development will empower multiple drug research applications, help fill future HTS pipelines and guide next generation combinatorial chemistry efforts.

INTRODUCTION

The Human Genome Project fostered a new approach to understanding the relationships between diseases and their underlying mechanisms. Equipped with genomic information and related technologies, scientists can now approach pharmaceutical research in a systematic and comprehensive manner. For example, the commercial availability of DNA microarrays has made routine the large-scale study of genetic variation and differential gene expression. However, while genomics has delivered many new targets, most of these targets are ill-characterized, and many are in fact poor targets. This is perhaps not surprising, as it is well known that proteins, not genes, are the targets of most drugs. Functional protein microarrays promise not only an important new capability to identify and characterize drug targets, but powerful new tools for downstream drug discovery and development.

The selection of 'druggable' targets for screening is the first, and in some sense most important, step in the drug discovery process. In order to efficiently identify and validate optimal therapeutic targets, scientists need to investigate potential targets in as much detail as possible. An exciting new capability for protein characterization is the development of functional protein microarrays; arrays of functional, positionally immobilized proteins. Miniaturization of these arrays enables the simultaneous study of thousands of proteins, a challenge which is otherwise impractical.

A wide variety of applications for protein microarrays has already been demonstrated. For instance, the ability to characterize interactions of proteins with a variety of biomolecules, including lipids [1], DNA [2, 3], carbohydrates, proteins [1, 2, 4-6] and small molecules [2, 7, 8], can be used to map protein pathways. Compared to current technologies, such as yeast 2-hybrid methods, protein microarrays have several distinct advantages, including the ability to make quantitative measurements [9]

and precise control of experimental conditions (see Fig. 1). The use of functional protein microarrays as 'substrate' arrays enables one to extend pathway analysis via rapid identification of enzyme substrates [7, 8, 10]. These combined abilities will ease the path towards more comprehensive understanding of protein pathways, and offer a powerful new approach to target discovery and characterization.

Applications are also being developed for functional protein microarrays which are further downstream in the drug discovery and development process. Initial reports are limited to proof-of-concept experiments, including the demonstration of fluorescently labeled FKBP12 [7] and G-protein coupled receptor [8] ligand binding to their respective receptors. However, as human protein content grows, direct applications to drug research will become more prevalent. For instance, the use of protein microarrays to profile small-molecule binding [7, 8] and/or activity has several powerful applications, including the identification of off-target effects, identification of alternate indications, 'rescue' of failed compounds and target characterization.

Functional protein microarrays have also been used to profile the specificity of antibodies, which has direct implications for the development and characterization of antibody therapeutics. Michaud *et al.* [11] recently demonstrated the use of protein microarrays for antibody specificity profiling. While the antibodies tested in this study identified their cognate antigen, they also identified unanticipated off-target binders. In addition to this study, smaller scale proof-of-concept experiments have been published [12]. The availability of a simple test for antibody cross-reactivity using properly folded proteins will simplify the development of antibodies for both research and therapeutic purposes.

Regardless of application, the widespread success and adoption of functional protein arrays will depend upon the ability to reproducibly manufacture large numbers of arrays tailored to specific applications. The purpose of this paper is to review the challenges and recent progress in the development of functional protein microarrays and protein microarray applications.

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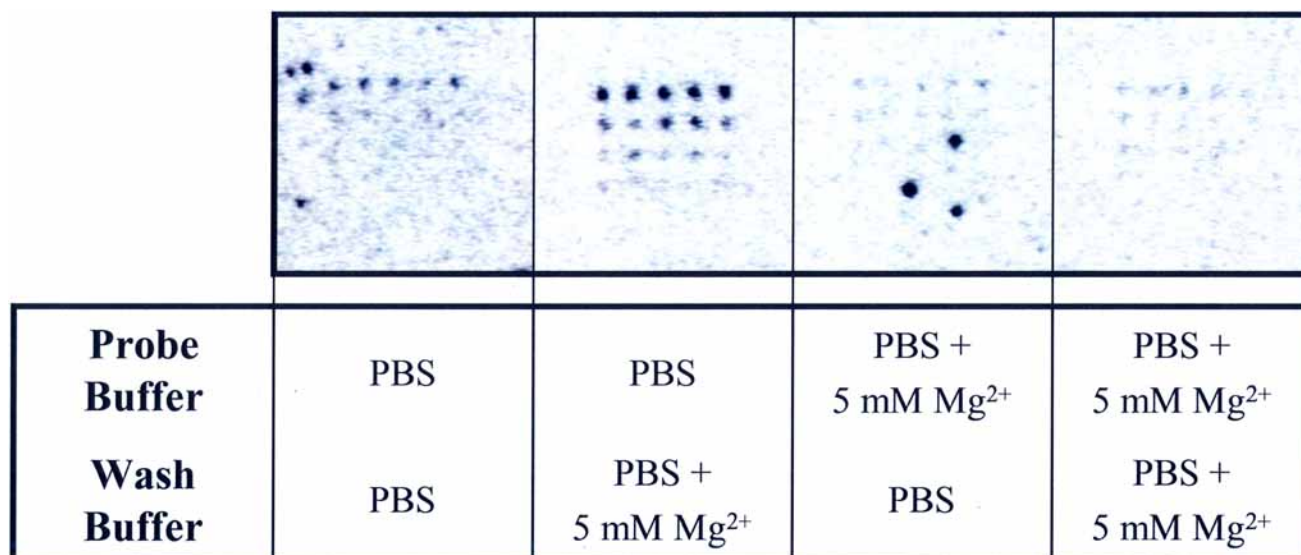


Fig. (1). Effect of buffer conditions on Ras binding to ³³P-GTP. Results from 4 different array probes under different probe and wash conditions are shown. Each experiment employed a concentration series of 5 spots of the protein Ras (concentrated to dilute, from top to bottom). Probe and wash conditions are specified on the bottom of the figure. The presence of Mg²⁺ in the wash buffer is required for sensitive interaction detection, presumably through slowing the dissociation rate of the GTP-Ras interaction.

DEVELOPMENT OF FUNCTIONAL PROTEIN MICROARRAYS

Users must understand the principles of protein microarrays in order to take full advantage of the technology and obtain optimal results. How do protein microarrays compare to conventional technologies? What are the similarities and differences? What new capabilities do protein microarrays offer, and how can users take maximal advantage of them?

Most of the challenges inherent in miniaturized and solid-phase assays are also important considerations for protein microarrays. Significant factors include diffusion, detection sensitivity, preservation of protein structure, protein function and surface chemistry. Given these complex requirements, commercialization of user-friendly protein microarrays will be required to make the technology widely accessible. Successful commercialization, however, will be contingent upon reasonable costs, simple procedures, compatibility with existing detection methods, instrument and software accessibility, quantitative reproducibility as well as rapid result generation and interpretation.

From the development perspective, protein microarrays are not a single technology. They comprise many technologies and require development in multiple areas. These areas include (1) protein content, which can be proteins generated either *in vivo* or *in vitro*; (2) microarray surface chemistry, for protein- and assay-compatible immobilization; (3) printing technology, which can be either pin-based contact printing or various non-contact methods; and (4) assay development, including labeling, detection and image analysis. Each of these areas impose significant challenges but also offer great opportunities for technology advancement. Commercial success will require a concerted effort integrating both technology and application development, with an understanding of the inter-relations of all of the related areas:

1. Protein Content

One of the most significant challenges in the production of functional protein microarrays is the generation of appropriate protein content. The major challenges include highly efficient and parallel 1) cloning, 2) expression, 3) purification and 4) quality control of proteins.

The first key step is the generation of large numbers of clones in expression vectors. Several efforts to develop targeted collections of ORF clones in a variety of systems have already been undertaken [13-15]. Most recent attempts to generate such clone collections have used vector systems with the ability to rapidly shuttle to different vectors for expression optimization [16].

The choice of protein expression system is a critical factor, and must balance high-throughput expression with protein functionality. For instance, while *E. coli* is a simple system for high-throughput expression, lack of many eukaryotic post-translational modifications makes the expression of functional human proteins less successful. On the other hand, eukaryotic expression systems are generally more appropriate for human proteins, but high-throughput expression can be challenging. Some classes of proteins, such as membrane proteins, may require specialized expression systems.

Parallel purification of large numbers of proteins requires a purification 'tag', a variety of which are in common use. The choice and location of tags can effect protein expression and function, often in ways not apparent prior to their selection. The availability of vector 'shuttle' systems, such as Invitrogen's Gateway® cloning system, greatly facilitates testing and optimization of expression constructs.

High throughput *in vitro* protein expression for manufacture of protein microarrays has recently been described. For instance, Kawahashi *et al.* demonstrated protein interaction detection using low complexity protein

microarrays manufactured from *in vitro* transcribed and translated protein [6]. Weng *et al.* employed a strategy to create mRNA-protein fusions combining *in vitro* translation with a modified mRNA conjugate (the PROfusion process) [17]. The resulting fusions can be hybridized to DNA molecules on a surface to generate a protein array. Scenarios which combine elements of expression, purification and even arraying can be envisioned. For instance, protein microarrays can be generated through *in vitro* expression of proteins *in situ*. Such approaches are not without their challenges, however. While *in situ* expression circumvents the need for separate expression and purification steps, reproducible generation of microarrays through such a process is likely to be extremely difficult.

Quality control of high throughput protein expression and purification processes is an important factor in array manufacture. In order to efficiently quantitate large numbers of different proteins, a common detection tag (not used for immobilization purposes) can be incorporated into every protein. With such a tag, fast and accurate measurement of protein concentrations becomes a simple process *via* automated procedures. After quantitation, it may or may not be desirable to normalize the concentration of all proteins on the array. Normalization of protein concentration enables a more straightforward comparison of signals originating from different proteins on the array. However, the normalization process can be time consuming and necessitates diluting proteins which would otherwise be present on the arrays at higher concentrations.

In addition to quantitation, proteins should be quality controlled for purity and integrity. The most straightforward way to assess purity and integrity is through denaturing polyacrylamide gel electrophoresis and general protein staining. A more sensitive measure of protein integrity is the use of denaturing polyacrylamide gel electrophoresis followed by Western blot against a tag expressed on the protein. The advent of commercially available gels has made both of these approaches more practical for large numbers of proteins, but still challenging due to lack of automation. While more complicated, mass spectrometry based methods may ultimately be more suitable for protein quality control.

2. Microarray Surface Chemistry

There are many commercially available microarray substrates, the majority of which are manufactured of glass in the form of microscope slides. The surfaces are either chemically modified or coated with a polymer layer to form a surface for protein immobilization. The immobilization characteristics and applications of different slides have been discussed in many recent reviews [18-20].

There are two basic mechanisms for protein immobilization: random and oriented attachment. In turn, each of these attachments types can be either covalent or non-covalent. In reality, the distinction between different immobilization methods can become blurred, as oriented attachment, for instance, can involve and even be dominated by random attachment mechanisms.

Some studies have suggested that oriented immobilization of proteins by specific interactions, such as biotin and streptavidin interaction, may offer better activity.

Peluso *et al.* biotinylated IgG and Fab' either randomly or at a specific site, followed by immobilization on a surface coated with streptavidin with near-monolayer saturation [21]. Using surface plasmon resonance and fluorescent detection on microarrays, immobilization efficiency and analyte binding studies indicated that the oriented Fab's had several fold higher binding-site density and specific binding activity than random ones. The oriented IgGs, however, gave only moderate increases in analyte binding, probably because the size and flexibility of full-length antibodies make the immobilization orientation less important. It should be emphasized that providing specific immobilization sites does not necessarily prevent random protein adsorption on the surface; proteins are free to interact with exposed surfaces even in the presence of specific sites. In practice, oriented immobilization may not give better results than adsorption methods.

A powerful strategy for oriented attachment of biotin-labeled proteins to avidin coated surfaces was recently published by Lesaicherre *et al.* [22]. In this method, proteins are expressed with a C-terminal intein. After expression, the proteins are purified on a chitin column. In the presence of biotinylated cysteine, the intein facilitates intramolecular rearrangements resulting in simultaneous biotinylation of the protein and its cleavage from the intein. The resulting protein, biotin-labeled at its C-terminus, can be bound to the surface of avidin functionalized glass.

Immobilization strategies need to protect proteins from surface denaturation as well as to preserve access to functional sites of the protein. Immobilization of proteins on hydrophobic surfaces may lead to denaturation and loss of function. In a functional study of $\beta 2$ adrenergic receptor ($\beta 2AR$), a ligand activated G-protein coupled receptor, Neumann *et al.* immobilized the protein on glass and observed ligand induced conformational changes by measuring a fluorescent label near its functional site [23]. To prevent direct interactions between the functional proteins and slide surface, slides were coated with biotinylated BSA, followed by streptavidin, essentially modifying the slide surface with proteins. Two similar immobilization strategies were then employed. The first used biotinylated anti-FLAG antibody, followed by a $\beta 2AR$ with a N-terminal FLAG tag. The second used a $\beta 2AR$ with site-specific biotinylation near its N-terminus. Both methods reproduced agonist-induced response in the receptor but the latter had lower sensitivity. The results suggest that although many proteins can be immobilized directly on a surface and remain functional, immobilization of other proteins, particularly membrane proteins, may need more sophisticated strategies to obtain optimal activity.

The immobilization efficiency or capacity of the slide surface is another significant factor in the development of protein microarrays. When considering either random adsorption or oriented attachment, the total available sites for binding, rate of immobilization, and stability of bound proteins during assay need to be compared.

Some slides, such as hydrogel and nitrocellulose membrane coated slides, offer higher binding capacity by creating a 3-D space, effectively large surface areas for protein binding. Angenendt *et al.* systematically studied 11 different surfaces for making antibody microarrays and found

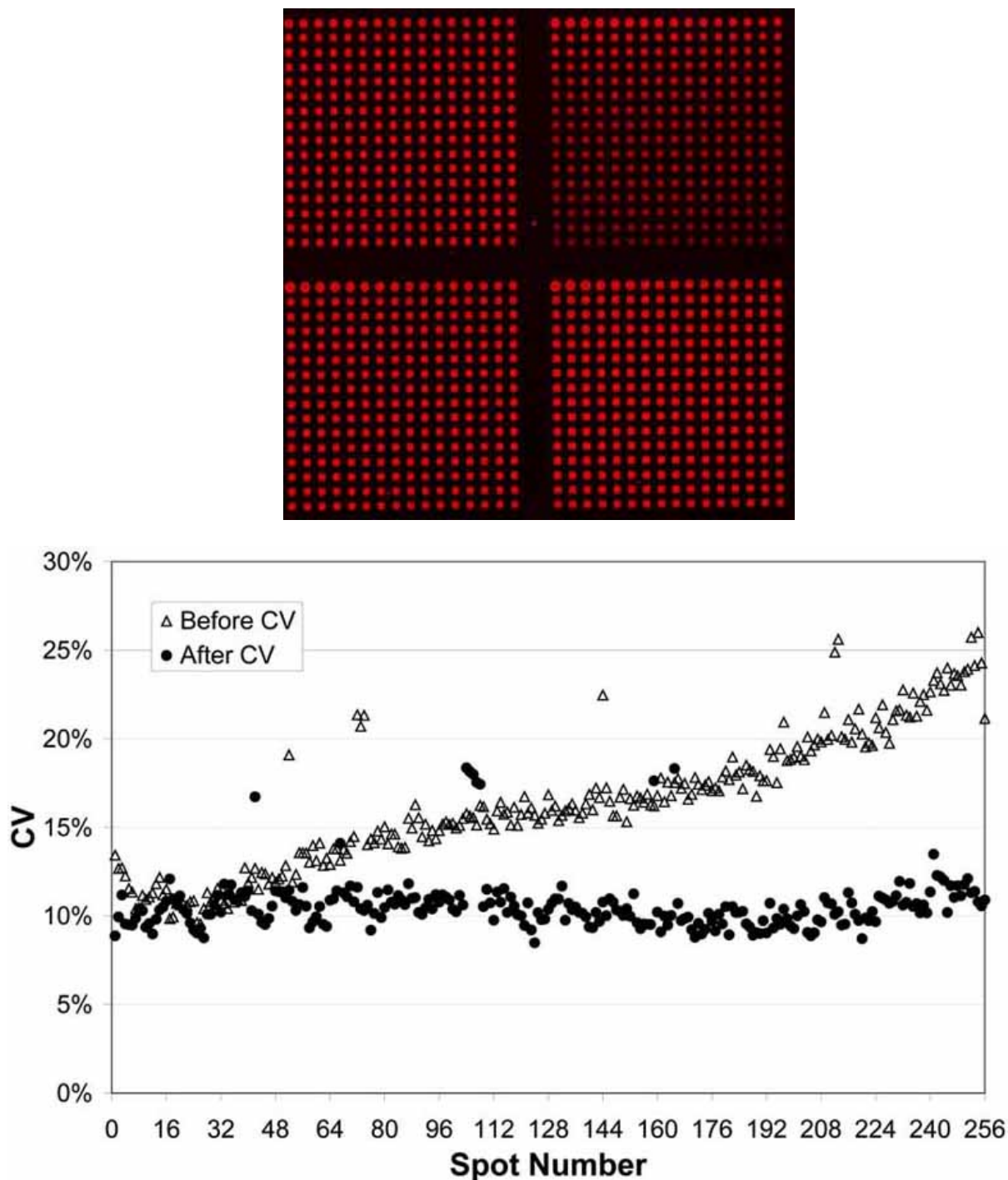


Fig. (2). *A.* Pin printing quality control (QC). The test slide was printed with fluorescent dye using 48 pins to create 48 subarrays. A zoomed in view of 4 subarrays is shown in the figure. The pin printing the upper right subarray failed QC, since it displayed poor sample delivery over the 256 spots in the subarray. *B.* Pin performance before and after pin replacement. The coefficient of variation (CV) for each pin (compared to all pins) is determined for each spot during the pin printing QC. The CV data is shown for one pin both prior to ('Before CV') and subsequent to ('After CV') replacement. This pin failed the initial QC due to substantial variation in CV during the print run, but passed QC after replacement. Constant monitoring of pin performance is required for reproducible manufacture of protein microarrays.

that polyacrylamide coated slides offered the greatest capacity and best sensitivity [24]. In addition to different detection limits of slides, they also found optimal signals after 2 weeks of storage for some slides, indicative of slow

immobilization kinetics on these surfaces. When the coefficient of variation (CV) was calculated for each surface, they found slides differed greatly, ranging from 11% to 31% for intrafield CVs and from 12% to 60% for interfield CVs.

The robust manufacture of derivatized slides remains a challenge, and surface heterogeneity is common, resulting in inconsistency in features on protein microarrays. When high consistency is required, extensive tests are needed to choose the optimal slides with the best immobilization characteristics and least surface variation. Further considerations of slide surfaces include their effects on spot size and density, interactions with blocking agents, probes and assay reagents, and compatibility with detection methods

3. Microarray Printing

There are several methods for microarray printing, ranging from experimental technologies [24-26] to commercially available options. Commercially available microarray printers can be classified as either contact or non-contact. Contact printing uses micromachined pins, normally made of metal alloy, that touch the substrate surface during printing. Non-contact printing employs ink-jet, piezoelectric, or similar technology to dispense samples without physical contact with the substrate. Some of the principles and examples have been discussed in recent reviews [26]. For proteome arrays, printer requirements include 1) design specifications suitable for operation at low temperatures (near 0°C), 2) environmental control of temperature, humidity, airborne particle levels, 3) fast and highly parallel dispensing and 4) compatibility with protein buffers.

Non-contact printers often have better precision and control but have fewer dispensers, and are not yet suitable

for printing high content protein arrays. Commercial contact printers with 48-60 pins offer the best choices in terms of throughput and reliability. A large number of pins increases the throughput but creates another challenge; calibration of all pins to produce minimal variation. Pins should be routinely tested to ensure acceptable printing variability, spot morphology and sample carryover. Rigorous manufacturing processes are required to enable reproducible results (see Fig. 2).

Printing layout is also an important consideration. When possible, proteins should be spotted in replicates to help add statistical significance to results. In addition, a variety of controls and calibration standards are useful. An example of a printing layout is presented in (Fig. 3).

4. Assay Development

Two basic types of assays are possible with functional protein microarrays. Interaction assays measure the binding of other biomolecules to proteins on the arrays, and enzyme assays measure the enzymatic activity of either an enzyme in solution (substrate assays) or of enzymes on the array (activity assays). Examples of the assays will be described, along with important considerations for assay development, including detection and analysis strategies.

4.1 Interaction Assays

Interaction assays have been demonstrated on protein microarrays using a variety of biomolecules, including lipids [1], DNA [2, 3], proteins [1, 2, 4-6] and small molecules [2, 7, 8, 27]. In each case, the basic assay protocol is highly

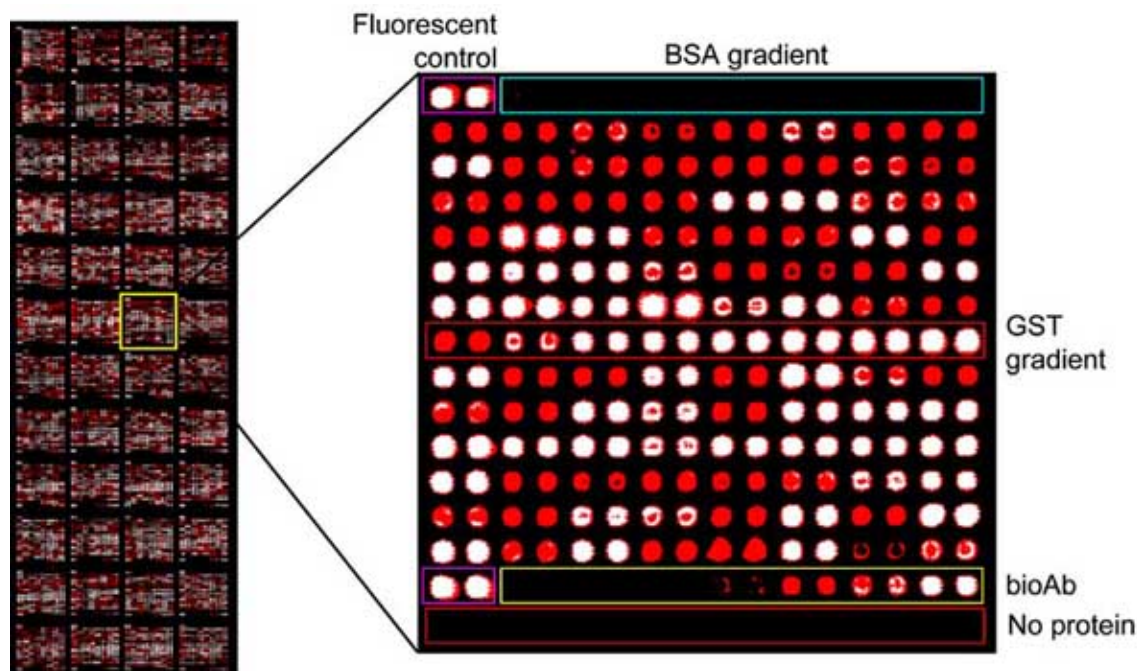


Fig. (3). Yeast proteome array image, and subarray layout (©2003 Protometrix Inc., reprinted with permission). A 48-subarray yeast proteome probed with α -GST antibody is shown on the left hand side. A single subarray is illustrated on the right hand side. Each subarray consists of a number of control and reference spots. All spots are printed in duplicate. Fluorescent control spots are used as reference points to help identify individual proteins on the array. A BSA gradient serves as a series of negative controls, and as a monitor of non-specific binding. A biotinylated antibody gradient is used as a positive control for probing with biotinylated molecules. A GST gradient can be used to generate a standard curve to estimate the amount of yeast proteins spotted throughout the rest of the array.

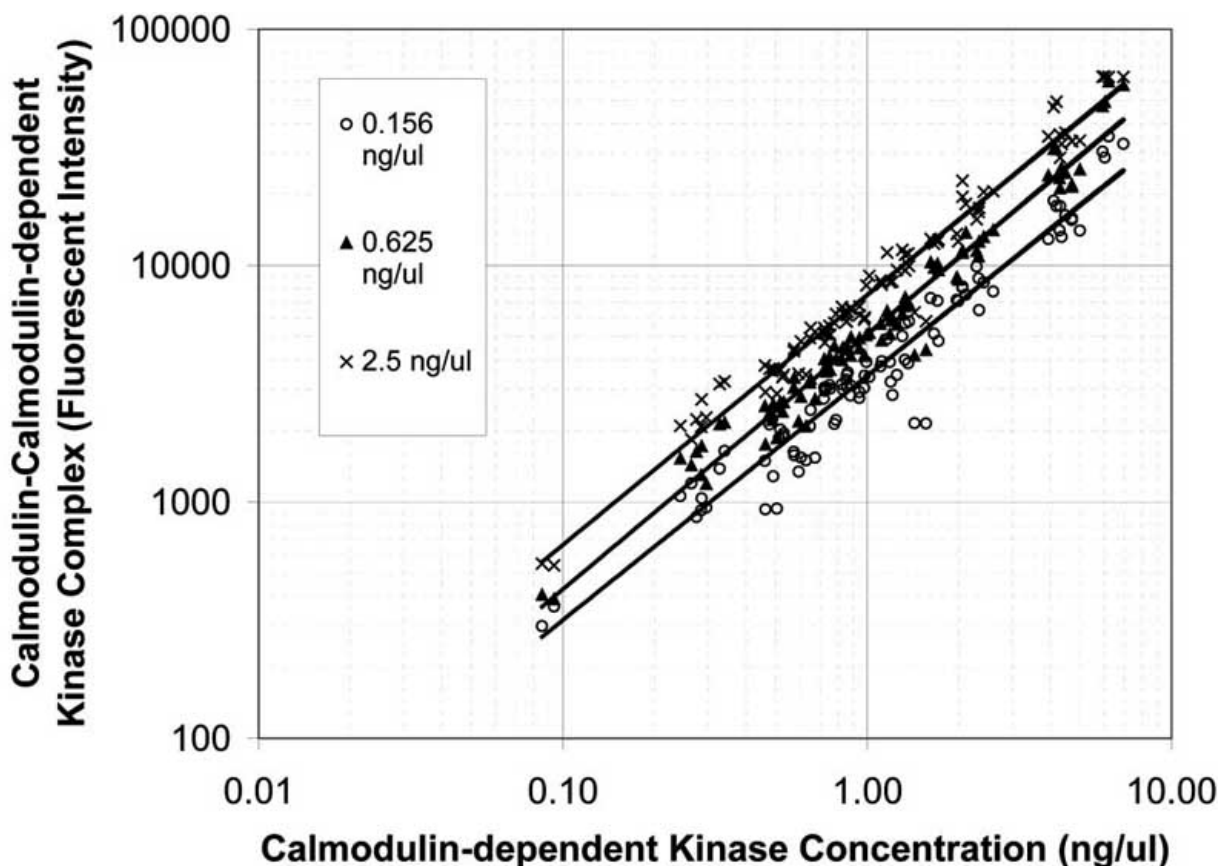


Fig. (4). Detection sensitivity. The plot shows the fluorescence detection intensity of a calmodulin:calmodulin-dependent kinase binding interaction. Microarrays were printed with varying concentrations of calmodulin-dependent kinase (x-axis) and were probed with biotinylated calmodulin at 3 different concentrations (indicated). Although signals are reduced, interactions can be detected even at very low protein concentrations.

similar ; block, wash, bind, wash, detect. The blocking step is important for reducing non-specific binding. The binding step allows for interaction between the probe and surface-bound protein. The subsequent wash step is important for removing loosely bound protein, although an excessively long wash can remove more tightly bound proteins as well, since off-rates, rather than equilibrium binding constants, determine the amount of protein removed. In some cases investigators have used multivalent probes in order to increase sensitivity by capitalizing on avidity effects [7].

The detection step is dependent upon the labeling scheme. A variety of strategies have been employed, including fluorescent labeling of the probe through direct chemical [4, 5, 7, 8], biochemical [2, 3, 6] or recombinant [28] means. In other cases proteins are recognized by secondary affinity molecules. While these molecules may be directly targeted to the protein, they are more commonly directed to tags encoded within the protein [29] (such as epitope tags) or to tags generated by protein modification [1] (such as biotin tags). Detection itself is dependent upon the labeling approach. Fluorescent labels of an appropriate wavelength can easily be detected [4-8, 11, 28, 30] using fluorescent microscope slide scanners, commercialized initially for mRNA expression profiling applications. Radiolabels have been used to detect interaction with

different probe types, including proteins [2, 29], nucleic acids [2] and small molecules [9]. In these cases, film or phosphorimagers are most commonly used for detection, although more specialized instrumentation may be used [31]. Chemiluminescence as well as colorimetric detection [32] can also be employed. Label-free detection strategies, such as surface plasmon resonance based methods, are less mature for high-density applications, and are not reviewed here.

Regardless of detection scheme, most assays face similar challenges. Sensitivity, for instance, is an important issue. Sensitivity may be increased through modified experimental conditions (including buffer, salt, pH, temperature, time or blocking conditions) or through signal amplification [33]. With proper attention to experimental conditions, interactions can be detected even when the amount of protein on the microarray spots are relatively low (see Fig. 4). Some radioisotopes, such as ^3H or ^{14}C , are very weak or tend to have relatively low specific activities which can be difficult to detect. In such cases, ensuring a high concentration of protein on the microarray can be helpful. Figure 5 shows an example of ^3H -based detection ; ^3H -GTP binding to G-proteins. Quantitative analysis of results can also be challenging. To start with, appropriate image analysis software is required to deal with the high data content of images generated during microarray experiments.

Fortunately, a large number of good image analysis software packages are available. If quantitative comparisons among probes or arrayed proteins is required, assay conditions also need to be performed in the linear response range. This generally requires repeated measurements at a variety of probe concentrations. Quantitative analysis is also dependent on intra- as well as inter-slide consistency. Although challenging, well controlled and reproducible manufacturing makes it possible to reliably determine apparent dissociation constants using microarrays [9].

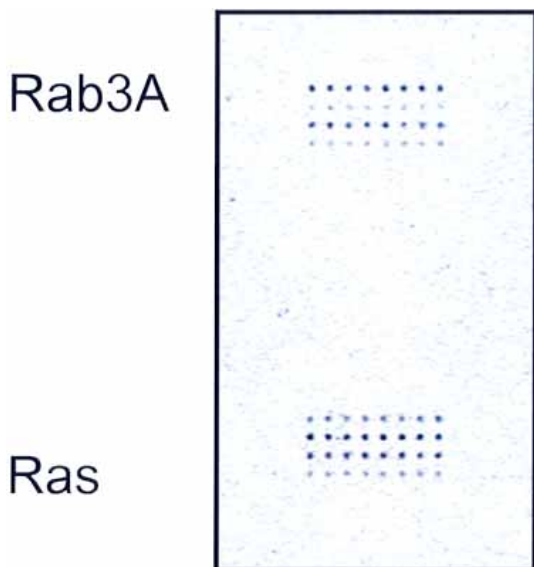


Fig. (5). Binding of ^3H -GTP to two G-proteins; Rab3A and Ras. Location of protein spots is indicated. Binding interactions were detected by a phosphorimager.

A significant practical challenge with interaction profiling against thousands of different proteins is establishing which interactions are significant. This difficulty is only exacerbated when proteins on the arrays are of varying concentrations. When large numbers of proteins are on the array, statistical approaches are the preferred method of establishing interaction significance. The Z-factor, a dimensionless normalized statistical measure of significance developed initially for assessing the significance of HTS hits [34], is well-suited to protein microarray analysis. A variant of this statistic was used in a recent study to identify significant interactions among coiled-coils on microarrays [4]. More straightforward methods can also be employed, such as establishing a minimum signal-background or signal/background threshold based on deviation from the mean [11]. At this point most published microarray experiments have employed relatively small numbers of proteins and have lacked rigorous statistical analysis. As high-content protein microarrays become commercially available, statistical analysis will become an increasingly important part of experimental analysis.

4.2. Enzyme Assays

Enzyme assays measure the enzymatic activity of either an enzyme in solution (substrate assays) or of enzymes on the array (activity assays). Substrate assays have been demonstrated for a variety of enzymes, including kinases [7] and methyltransferases [10]. Readily accessible detection methods for substrate assays include radiolabels [7, 10] and

secondary detection by antibodies [35] or fluorescent stains such as Molecular Probes' phosphoprotein detection reagent Pro-QTM. In an interesting variation on substrate assays, Chen *et al.* have pioneered the use of mechanism-based inhibitors to detect enzyme activities on microarrays [36]. Regardless of the exact protocol, substrate assays performed on arrays with thousands of proteins provides a rapid and powerful new approach to identifying enzyme substrates.

Microarray-based activity assays present a potentially powerful approach to study the effects of inhibitors on large numbers of enzymes simultaneously. An early demonstration of such assays include the measurement of horseradish peroxidases, alkaline phosphatase and β -D-glucuronidase activities [32]. This study also reported an ability to measure kinetic parameters and enzymatic inhibition. Unfortunately, the methods are not easily generalized, as they employed the use of precipitating reaction products. Even so, they present a powerful demonstration of an important new capability in development for small molecule screening.

SUMMARY AND FUTURE PROSPECTS

Although some investigators have demonstrated basic feasibility, creating highly consistent microarrays with thousands of proteins remains a daunting task. Developing reliable and user-friendly protein microarrays for uses beyond proof-of-concept is a highly complex process that requires a concerted effort, but is crucial to widespread use of the technology. To realize the maximal potential of protein microarrays, users and developers alike need to understand the entire process and its limitations, and strive to overcome technical challenges in a number of closely related areas. Ideally, these challenges should be addressed in a systematic and integrated fashion; or else an improvement in one area may lead to problems in the others. Successful commercialization of the technology will enable widespread adoption for multiple applications and, eventually, the development of applications which are not yet even conceived.

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