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# Protein microarrays: prospects and problems

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**Abstract**

Protein microarrays are potentially powerful tools in biochemistry and molecular biology. Two types of protein microarrays are defined. One, termed a protein function array, will consist of thousands of native proteins immobilized in a defined pattern. Such arrays can be utilized for massively parallel testing of protein function, hence the name. The other type is termed a protein-detecting array. This will consist of large numbers of arrayed protein-binding agents. These arrays will allow for expression profiling to be done at the protein level. In this article, some of the major technological challenges to the development of protein arrays are discussed, along with potential solutions. 

**Keywords:** Protein array; Microarray; Protein–protein interaction; Protein immobilization; Surface plasmon resonance

## 1. Introduction

Nowhere is the frenetic pace of biological research more apparent than in the genomics/proteomics area. Only this summer, the human genome sequence was (mostly) completed, an event that many compared in significance to landing a man on the moon. Yet almost before the ink on the *Time* magazine cover was dry, it seemed that most of the discussion had already turned to the next huge challenge: building on this wealth of DNA sequence information to 'solve' the human proteome. Most biochemists would define this formidable undertaking as characterizing every protein encoded by the human genome. This would include understanding its function, structure, molecular interactions and regulation in various cell types. It is likely that chemical biologists can make major contributions to this area.

In order to tackle a problem of this magnitude, it is clear to everyone that new techniques capable of very high throughput will be required. High on this wish list are so-called protein chips or protein microarrays, of which there are two types. I would like to propose a nomenclature to distinguish these, since their applications and the challenges involved in constructing them are very different. One is an array in which each protein in a cell occupies a defined spot on the chip. I will refer to these as protein function arrays since such devices would be employed for highly parallel studies of the activities of native proteins. For example, if one wished to know all proteins associated with some protein X, then a fluorescently labeled protein X derivative might be incubated with a protein function microarray. Spots that 'light up' would be considered excellent candidates for protein X binding partners (Fig. 1). The second type of chip is more correctly called a protein-detecting array. In this case, rather than spotting down the native proteins themselves, one would array highly specific ligands to each protein capable of recognizing their target polypeptide in complex biological solutions, such as a cell extract. This type of chip would serve as an analytical tool somewhat analogous to DNA microarrays in the sense that it would be capable of monitoring protein levels in a given biological sample in a massively parallel fashion (Fig. 1). In other words, protein-detecting arrays would be to the Western blot what DNA microarrays are to the Northern blot. The potential impact of protein-detecting chips extends far beyond basic research. It seems likely that if this technology could be made practical, it might form the backbone of 21st century medical diagnostics. Given the huge potential market for such devices, industrial interest in protein-detecting chips is very high.

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This article reviews progress in this very young field and discusses some of the challenges that must be surmounted to achieve practically useful devices. Before beginning, it is important to admit that there is undoubtedly important but unpublished work going on of which I am unaware, particularly in the biotechnology industry. Thus, this discussion should not be considered a comprehensive review of this rapidly expanding area.

## 2. Why do we need protein function and protein-detecting arrays?

Whenever one wishes to develop new methodology, whether in organic synthesis, molecular biology or proteomics, it is critical to first address the limitations and problems of existing related technology and to focus new efforts on addressing these issues. The 800 lb gorilla in the high-throughput genomics area is the DNA microarray, which has revolutionized many areas of biology [1]. DNA microarrays are chemically modified glass slides onto which have been placed large numbers of different DNAs that are complementary to nucleic acids of interest, for example genes. Depending on the type of array, these DNAs are oligonucleotides synthesized on the chip by photolithographic methods [2], or polymerase chain reaction (PCR) products spotted onto the chip by a robot [3]. The application of DNA microarrays most relevant to this article is to monitor the effect of a given stimulus or genetic alteration on the expression of thousands of genes simultaneously (genome-wide expression analysis). Without going into technical details, the basic protocol is to isolate total mRNA from the sample of interest and to make cDNA by reverse transcription (RT). Fluorescent labels are incorporated during this process, allowing later visualization of the DNA. The sample is then hybridized to the DNA microarray under conditions where there is far more immobilized probe than DNA analyte. Thus, the intensity of the fluorescence that hybridizes to each spot reflects the amount of each mRNA molecule in the original sample. By comparing data obtained from two different samples, for example wild-type yeast and a congenic deletion mutant, or cells that were or were not treated with a given drug, illuminating insights can be obtained regarding global effects of these variables on cellular metabolism. One of the more striking applications of this technology has been the discovery of gene expression patterns characteristic of a disease state, such as cancer [4,5].

As useful as DNA microarrays are, they have several limitations. Of course, what one would really like to know is how a genetic alteration, drug, or change in the environment effects protein levels and activities. mRNA-based measurements provide only an indirect measure. The expression levels of many genes are subject to significant post-transcriptional regulation, meaning that the message levels do not always reflect protein levels accurately. Sometimes this discrepancy can be 20-fold or larger [6]. Even more important is that the activities of many proteins are grossly affected by post-translational modifications such as phosphorylation, glycosylation, acetylation, proteolysis, etc. Obviously, a nucleic acid-based array is blind to such effects. Finally, for certain applications, the tedious sample preparation requirements of DNA microarrays make them impractical. For example, because of heightened concern over bioterrorism, the US Department of Defense is very interested in developing new technologies with which to detect pathogen infections. In theory,

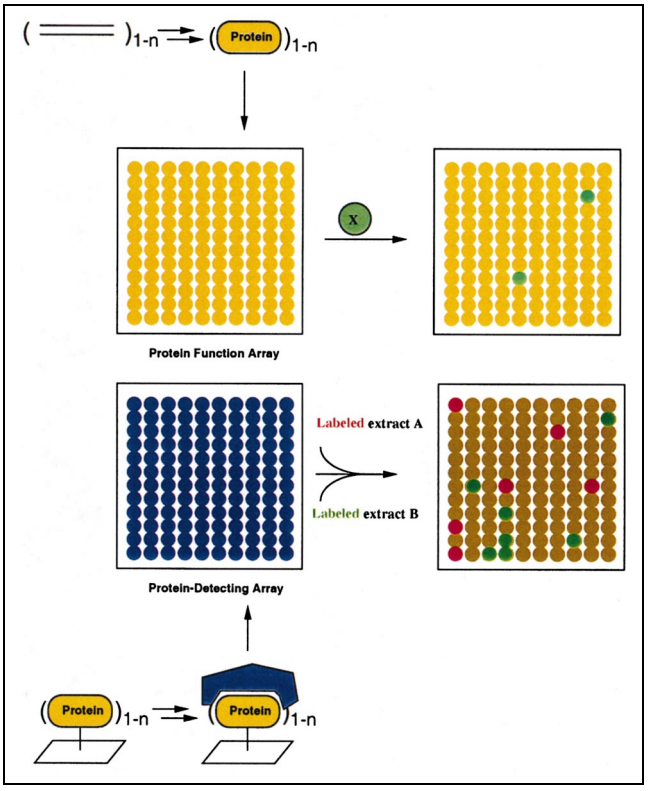


Fig. 1. Schematic representation of the two major classes of protein arrays. Top: Protein function arrays. Native proteins (colored orange) will be obtained from cDNAs by in vitro transcription/translation or some other technique, then spotted down onto a suitable surface. These arrays will be used to probe the function or binding properties of native proteins. In the example shown, the array is sampled for those factors that bind directly to a particular protein X, that is labeled with a green dye such as fluorescein. The spots that 'light up' are candidate binding partners. Bottom: Protein-detecting arrays. In this case, the native proteins are used as targets for library screening experiments. One of more ligands (shown in blue) that bind with high affinity and specificity to each protein will be obtained. These protein ligands will then be arrayed on the appropriate surface. These chips will be used for proteome profiling. In the example shown, all of the proteins in two biological samples that one would like to compare are labeled with distinguishable markers (here represented as red or green colors). The mixed samples are then incubated with the array. Spots that show up as green or red have an excess of protein from one sample over the other. Spots that appear brown are meant to represent a rough equivalence of the amount of proteins from each sample. This type of experiment, analogous to gene expression profiling with DNA microarrays, would allow the effect of various physiological stimuli or genetic alterations on proteins levels to be examined.

DNA microarrays could be very useful for this purpose, since even an early stage, pre-clinical pathogen infection would probably produce a diagnostic signature pattern of gene expression in certain cells. However, for this kind of real world application to be practical, thousands of individuals would have to be tested rapidly using readily obtained and minimally processed samples. Thus, the RT/fluorescent labeling technology required in current DNA microarray technology makes it a non-starter for the military. One can imagine that similar issues, but based on cost rather than speed and convenience, might limit the general use of DNA microarrays in civilian medical diagnostics.

The solution, of course, is to analyze proteins directly, rather than make inferences based on RNA levels. This is perhaps the key goal in the new area of proteomics. Proteomics technology is currently dominated by two-dimensional (2D) gel electrophoresis/mass spectrometry (MS)-based methods [7]. In this approach, the proteome of interest is resolved as much as possible into discrete protein spots using 2D electrophoresis and then the identity of the protein(s) in each spot is identified by MS or MS/MS. While useful for certain applications, this technique is very limited in scope. MS is an excellent detection tool, but cannot easily be used quantitatively, though novel strategies are being developed to circumvent this problem (vide infra). The real limitation here though is the 2D electrophoresis technique. Obviously, this tedious process cannot support the high-throughput required of real world medical diagnostics. Even more seriously, careful recent studies have shown that low abundance proteins are not easily detected in silver-stained 2D gels. For example, a study using yeast extracts concluded that almost half of the proteome was invisible using 2D electrophoresis/MS proteomics [8]. This is unfortunate, since it is often the less highly expressed proteins that respond in the most interesting way to various physiological stimuli. Therefore, while 2D electrophoresis/MS-based methods will probably continue to be the workhorse in the field for the next couple of years, the need to move beyond this technology is clear.

The above discussion was mostly relevant to protein-detecting arrays. Why do we need protein function arrays? The aforementioned example of detecting all binding partners of a given protein X is a good place to start. Most investigators would employ one of two techniques for this purpose, either a two-hybrid experiment [9,10] or protein affinity chromatography [11]. The former is extremely useful, but suffers from the fact that the proteins of interest must be employed as part of artificial fusion proteins. This means that binding assays in which one wishes to employ a multi-protein complex, for example, cannot be carried out using this method. In addition, proteins that do not express well in yeast or which are transcriptional activators cannot be employed as bait in this assay. Perhaps most importantly, the control of the experimenter over the conditions employed in an in vivo assay is very limited, while in a protein function array-based experiment, the conditions could be adjusted as desired. The array-based method is distinguished from affinity chromatography in that it would only detect direct binding events. A unique application of protein function arrays would be to investigate small molecule–protein interactions on a proteome-wide scale, for which there are not many good methods [12]. This type of application is relevant to a problem frequently encountered in the pharmaceutical industry, which is whether a given drug candidate binds tightly to any proteins other than its intended target. This sort of analysis would be relatively straightforward using a protein function array and a labeled drug candidate.

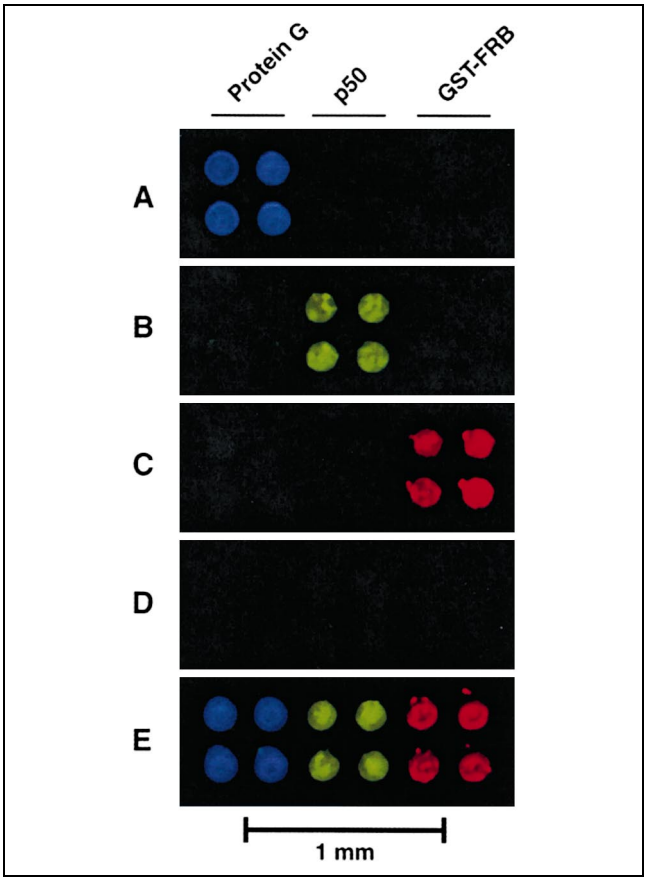


Fig. 2. Example of a model protein function microarray and its use in probing protein–protein interactions. Protein G, p50 and the FKBP-rapamycin binding (FRB) domain of FRAP were spotted onto a chemically derivatized glass slide, four spots each. The slide was then probed with various fluorescently labeled proteins. (A) Slide probed with BODIPY-FL-IgG, known to bind protein G. (B) Slide probed with Cy3-labeled IkBa, known to bind to p50. (C) Slide probed with Cy5-labeled FKBP12, known to bind FRB in the presence of rapamycin. In this experiment rapamycin was included in the buffer. (D) Same as (C), except rapamycin was not present. (E) Slide probed with all three labeled proteins in the presence of rapamycin. BODIPY-FL, Cy3 and Cy5 fluorescence were false-colored blue, green and red, respectively. Reprinted with permission from [13].

## 3. Near-term prospects for protein function arrays

The construction and use of protein function arrays is a much simpler problem than the development of practically useful protein-detecting arrays. Indeed, significant progress has recently been reported in the former area. MacBeath and Schreiber have described the immobilization chemistry and robotics necessary to make high-density protein function arrays [13]. They also reported model experiments that indicate that these arrays should indeed be useful for the sort of binding experiments discussed above (Fig. 2). The main issue in moving from this demonstration of feasibility to real protein function arrays will be to produce the thousands of proteins to be spotted down onto the slide. For complete protein function arrays (i.e. covering the entire proteome), it is probably impractical to do this by standard methods of expression of tagged recombinant proteins in *Escherichia coli* or Sf9 cells followed by affinity purification, though for modest-sized arrays this would be reasonable (also see [14] for a pool-based method using glutathione *S*-transferase fusion proteins). More likely, in vitro transcription/translation methods will be used, since only small amounts of proteins are required. Several large, arrayed clone collections are available commercially. These could be employed as templates for PCR using primers designed to incorporate a promoter and an affinity tag, thus producing the DNA substrate for in vitro transcription (Fig. 3). Subsequent in vitro translation would then produce the tagged protein which could be immobilized selectively at the appropriate spot on the array by specific binding to a capture agent. The simplest example of this would be to epitope-tag the protein and display the cognate monoclonal antibody on the slide. Alternatively, a protocol has been developed to incorporate biotin co-translationally into a protein (Hayhearst et al., personal communication; also see [15]). The least desirable, but potentially workable, solution would be to perform a quick, automated affinity purification of the tagged protein and then bind it covalently to a chip modified with lysine-reactive groups.

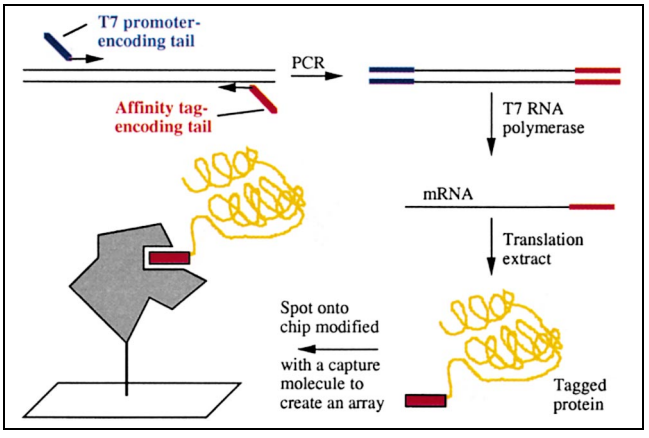


Fig. 3. Diagram of a protocol that could be employed to make protein function arrays comprised of thousands of immobilized proteins. PCR would be employed to amplify the gene of interest from cDNA using primers that would allow a T7 promoter to be incorporated 5′ to the gene and which would place sequences encoding an affinity tag at the 3′ end. In vitro transcription/translation would provide the desired protein containing the affinity tag. The protein would then be immobilized by spotting onto a glass slide impregnated with a molecule able to bind the affinity tag tightly. All other proteins in the translation mixture would be washed away. Many tag/capture pairs can be imagined. The figure depicts a small epitope (red box) that is recognized by a monoclonal antibody.

This is one reason that protein function arrays are much simpler than protein-detecting arrays. It is obvious what to spot down. The other is that for at least first-generation applications of protein function chips, a single or a small number of soluble analytes will be employed. These can be labeled synthetically, allowing ready visualization of the positions on the chip at which they bind. In the case of protein-detecting arrays, it is not obvious what to spot down and fluorescent labeling of the cell extract, blood sample, etc. may not be an optimal approach to visualizing binding events. In summary, it seems likely that in the near future, protein function arrays will become widely available and will occupy an important place in the battery of techniques by which to analyze molecular interactions on a genome-wide scale.

## 4. Protein-detecting arrays: the ligand problem

The ideal proteomics-based analytical tool would consist of a microarray of a large number of high affinity, high specificity protein ligands, one for each protein in the proteome of interest. For humans, this would mean isolating in the order of 100 000 good monoclonal antibodies or the equivalent (vide infra). In reality, the number would be much higher, since the detection of different post-translationally modified forms of a protein is one of the principal advantages of moving from nucleic acid to protein-based analytical techniques. Just to take a round number, let us assume that 1 000 000 high affinity ligands would be needed to make the ideal protein-detecting microarray. Would anyone care to isolate that many monoclonal antibodies? Of course, the field will proceed in a stepwise fashion and a reasonable intermediate goal would be to construct arrays of 1000 protein-binding ligands directed against proteins of interest in a particular disease state, for example. Nonetheless, these numbers highlight the first problem that must be solved to begin to seriously develop protein-detecting microarrays: the rapid and efficient isolation of high affinity and specificity protein ligands. While the first protein-detecting chips will almost certainly be based on monoclonal antibodies produced by traditional means, these will largely be for show. It is simply impractical in the long run to make many thousands of monoclonal antibodies by standard protein preparation/animal immunization/hybridoma techniques.

This is not to say that antibodies are not promising

binding agents for use in protein-detecting arrays (for example, see [16]). On the contrary, they are currently the only class of molecules generally able to serve as high specificity, high affinity ligands for almost any given protein. The challenge will be to devise alternative methods for their isolation that are amenable to high-throughput and scale-up. For example, libraries of single-chain antibodies can be displayed on the surface of bacteriophage, bacteria or yeast and these libraries can be panned for molecules that bind to a given protein target [17–21]. There a number of important technical problems to be addressed before high affinity antibodies can be obtained in a high-throughput fashion, but this route to recombinant antibodies is likely to be a major player in the construction of first-generation protein-detecting arrays.

Competing with recombinant antibodies will be various antibody-like (in a functional, not structural, sense) molecules that have been termed protein aptamers. These are protein derivatives engineered to display peptide libraries. Perhaps the best examples of such species are the thioredoxin-based aptamers developed by Brent et al. [22–24]. Thioredoxin is a small, very stable protein that contains a surface loop that is highly tolerant of sequence variability. At the nucleic acid level, a peptide library-encoding mixed oligonucleotide library was inserted into the appropriate position of the gene to create the aptamer library. This was done in the context of a fusion to a transcriptional activation domain. This allowed the library to be screened by a modified two-hybrid method in yeast using a target protein fused to a sequence-specific DNA-binding domain. Very good results have been reported using this system. Aptamers capable of binding their targets with $K_D$s in the nM region were obtained in several cases.

Another possibility is nucleic acid aptamers, either RNA or DNA [25]. Nucleic acid aptamers can be selected in vitro based on repeated cycles of binding and PCR. A major advantage of this class of molecules is that it is very straightforward to carry out affinity maturation in the process of the selection, allowing very high affinity ligands to be obtained [26]. Given that they are highly negatively charged molecules, it is likely that nucleic acid aptamers will work well with targets containing positive charge patches on their surface. How broad their applicability will be in a proteome-wide sense remains to be determined.

Finally, one can consider small molecule ligands for proteins. Since the term small molecule means different things to chemists and biologists, I will define it to mean a species that can be obtained readily by chemical synthesis. In the long run, small molecule ligands have enormous potential advantages over macromolecular protein or nucleic acid ligands. They are generally far more robust. More importantly, one can carry out chemical synthesis on large scales, thus making supply of the protein-binding ligands much simpler if such technology were scaled up to commercial levels.

It is now becoming commonplace to identify organic compounds that bind proteins through combinatorial library methods. In general, a library is made by solid-phase synthesis using split/pool methodology and, if necessary, encoded tags are included in the synthetic scheme. The collection of beads is then mixed with a labeled protein derivative and the beads which bind the protein of interest are identified. Usually, this process is carried out with a single protein target, but there is no reason why it could not be made more parallel by employing many targets at once. Fig. 4 depicts a scheme that represents the limit of this sort of approach. One could take a cDNA library designed to express a fusion of the proteins of interest to green fluorescent protein (GFP), horseradish peroxidase (HRP) or some other easily detectable molecule and transform this library into *E. coli*. Rather than segregating the resultant transformants, all of the bacteria would be grown up together and a mixed extract would be made containing all of the GFP fusion proteins (or at least all of those that express well in soluble form). This mixture could then be incubated with a library of bead-bound chemicals. After the appropriate washing steps, any beads that light up would be collected, resulting in the isolation of all compounds in the library that have a reasonable affinity for some protein in the proteome of interest. Which compound binds which target would then have to be deconvoluted. This might be as straightforward as sampling the fusion protein bound to each bead by MS.

It seems likely that some strategy akin to that shown in Fig. 4 will be capable of providing a wealth of lead compounds. The real challenge in developing small molecule-based protein-detecting arrays is in isolating ligands with high affinity. It has been our experience, working with

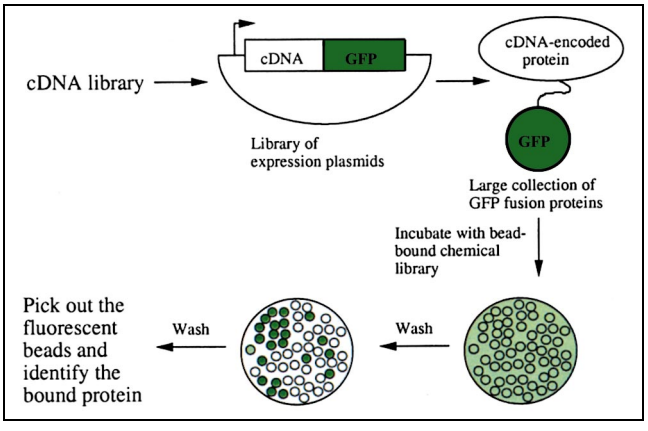


Fig. 4. A scheme for screening chemical libraries against many protein targets simultaneously. A library containing many labeled proteins (in the case GFP fusions) is expressed and the mixture incubated with a bead-bound chemical library constructed by split/pool synthesis. After washing, the beads that remain fluorescent are collected and scored as possible protein ligands. Some scheme would be required to deconvolute which protein bound to any particular bead. This might be done by direct MS analysis of the material on the bead. The library would have been pre-screened with GFP lacking a cDNA-encoded fusion. Any GFP-binding beads would be removed prior to the experiment.

peptide libraries, that the best ligands one isolates in such screens bind their targets with $K_D$s in the order of $10^{-6}$ to $10^{-7}$ M [27,28]. While these modest affinities may be suitable for some applications, one would ideally like to have ligands that bind their target with the affinity of a good antibody ($K_D$ in the $10^{-9}$ to $10^{-12}$ M range). In general, it seems clear that the lead molecules will have to be elaborated significantly to achieve large jumps in affinity. It is unlikely that methyl/ethyl/propyl-level tinkering will achieve this goal and traditional SAR/affinity maturation approaches are much too tedious in any case. One promising approach is to link together two low affinity ligands that bind different surfaces of a given target, but current methods [29,30] to execute this strategy will have to be reengineered to accommodate a much higher throughput. This is certainly an area where a creative chemical biologist could have an enormous impact. One advantage here relative to the somewhat analogous development of a drug from a lead compound is that the molecular mass, cell permeability and other pharmacokinetic properties of the protein ligand are irrelevant as long as large quantities can be made easily at the end of the day.

## 5. Protein-detecting arrays: the detection problem

As difficult as it is going to be to isolate and produce thousands of high affinity and specificity protein ligands, it may be even harder to come up with a good way to monitor binding of proteins to the chip. Thinking in this area is dominated by analogies to DNA microarrays, which may not be very useful. As mentioned above, the cDNAs that are actually detected in an expression analysis experiment are fluorescently labeled during the reverse transcription step. Obviously, the analogous protocol cannot be used to label a protein, but it is generally assumed that one could fluorescently label proteins chemically. For example, one could take a cell extract and treat it with a commercially available *N*-hydroxysuccinimide ester of carboxyfluorescein or some similar compound to derivatize the terminal amine of lysines that are commonly on the surface of most proteins. This sample would then be incubated with the chip and the retained fluorescent proteins would be detected with a simple reader as is now done in the DNA microarray field.

There are several problems with this approach, though it will probably be the one adopted in the early days of protein-detecting microarrays. First, the chemical heterogeneity of proteins makes this hopeless as a strategy for doing quantitative work. Some proteins will label far more efficiently than others. The absolute intensity observed on a particular spot on a chip would be meaningless in the absence of a calibration curve determined using well-characterized standards. Again, the scale involved defeats the idea of generating calibration curves for every protein–ligand complex. However, it should be pointed out that even in the DNA microarray area, absolute quantitation is difficult and most experiments focus on discerning differences between two samples. Most protein-detecting arrays will likely be employed in the same way (see Fig. 1, bottom). For example, extracts made from wild-type yeast and a congenic deletion mutant would be treated with two different colored dyes. The extracts would be mixed, then incubated with the protein-detecting array. The ratios of colors that bind to each spot on the array would be recorded, in direct analogy to DNA microarray methodology, providing a relative measurement of the change in expression level of the various proteins. In theory at least, this would render the difference in the chemical efficiency of fluorescence labeling of various proteins unimportant.

With relative abundance experiments in mind then, the real problem with chemical labeling of proteins is that it changes their surface characteristics greatly. In particular, reactions that convert positively charged lysine side chains to amides could result in significant protein denaturation. Even more troubling is that the fluorescently labeled protein may no longer bind to the immobilized ligand, which was selected against the native protein. This will be an especially large concern with smaller proteins and peptide hormones. It remains to be seen how pervasive a problem this will be, but chemical labeling of the protein sample is clearly not the optimal solution for the visualization of binding events. Even if the above concerns prove to be overstated, chemical derivatization represents an unwanted and labor-intensive processing step that would make the high volume use of protein-detecting arrays more problematic.

A major opportunity in this area in to devise a solution to the detection problem that does not require sample labeling. In general, the most appealing solution would be to modify the capture ligands with some sort of sensitive reporter that would record the analyte protein-ligand binding event. There are many possibilities, but perhaps the most appealing would be to attach the capture ligands to materials that can change their emission or conductive properties significantly in response to a binding event. This would provide an intrinsically responsive surface. An alternative would be to modify the capture ligands with a reporter capable of signaling binding of the target of the macromolecule with high sensitivity.

Another general type of approach would be to employ a sandwich assay of some sort (Fig. 5) in which the analyte protein of interest is bound by both the immobilized capture ligand and a soluble sandwich ligand. The latter would be equipped with some reporter that is easily detectable. The labeled sandwich ligand would adhere to the chip only if the protein analyte was bound. An advantage of a sandwich approach is that the sandwich ligand could be conjugated to an enzyme (Fig. 5), thus allowing significant amplification of the binding signal. This will be very important for the detection of low level proteins, hormones, cytokines, etc. The obvious downside to this oth-

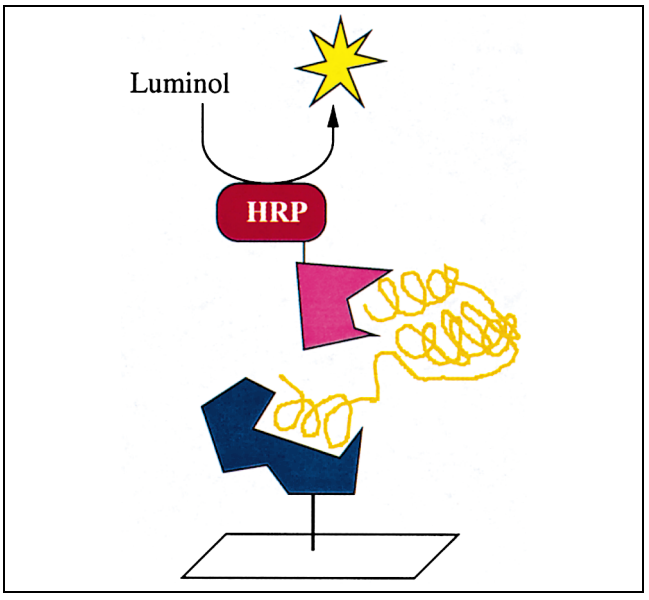


Fig. 5. Schematic view of a sandwich assay for detecting binding of the target protein (orange) to the immobilized ligand (blue). A second protein-binding agent (pink) would be isolated that does not compete with the immobilized capture ligand. The latter molecule would be fused to HRP (in red) or some other reporter enzyme that would allow amplification and visualization of the binding event. This sort of detection strategy would not require chemical labeling of the protein sample.

erwise appealing scheme is that two, rather than one, ligands are required for each protein. This would not be so bad except that one must know that the capture and sandwich ligands do not compete for the target protein. This means that either competition binding experiments would have to be conducted with ligands isolated in an initial screen, or that a second screen would have to be conducted for ligands that bind the protein of interest in the presence of saturating amounts of the initially selected ligand. In either case, this further complicates an already daunting ligand isolation problem.

There are some existing protein analytical techniques with enough sensitivity to detect binding of proteins to the immobilized ligands directly without the requirement of labeling or amplification. The most obvious is MS. For example, one can imagine immersing a protein-detecting array in an appropriate matrix, then sampling each spot on the array by matrix-assisted laser desorption-ionization time-of-flight MS. The Achilles heel of MS is that it is difficult to quantitate signals, so it would be challenging to compare the intensities of the protein signals from corresponding spots on two different chips. Of course, one cannot mix two different samples and apply them to a single chip, since the signals from the same protein in the two samples will be indistinguishable. To address this problem, Aebersold et al. have recently reported an isotope labeling technique that is somewhat analogous to the idea of two-color labeling, but employs isotope tags rather than fluorescent dyes [31]. A reduced sample is treated with a sulfhydryl-reactive, biotin-containing agent. The proteins are then proteolyzed and the derivatized, cysteine-containing peptides are collected by avidin affinity chromatography and identified by high performance liquid chromatography-coupled MS/MS. The trick here is to treat one of the two samples to be compared with a $D_8$-derivative of the cysteine-reactive compound. This provides an isotopic label that allows peptide from one sample to be compared quantitatively to the analogous species obtained from the other sample. This technique has many of the same drawbacks as fluorescent protein labeling and is not readily applicable to the format of a protein-detecting array. However, I bring it up to suggest that if some clever technique could be devised by which one could isotopically label proteins in vivo, then direct MS analysis of proteins from two samples bound to the chip could be carried out quantitatively.

Finally, binding events could be determined by surface plasmon resonance (SPR) [32]. While technically incorrect (I will spare the reader a digression into the physics of plasmon resonance), it is pedagogically useful to think of an SPR instrument (better known to many biochemists as a BIAcore) as a highly sensitive microbalance in which a laser interrogates the backside of a chemically modified gold chip and records changes in the mass of the immobilized complex. SPR is a very sensitive technique and appears quite attractive for this application. The technical hurdle here is that an SPR instrument would have to be made that is capable of analyzing thousands of spots in a relatively short period of time. While I am not an engineer, it seems reasonable that such an instrument could be constructed by allowing the laser to move from feature to feature (or more probably moving the multi-feature chip with respect to a stationary laser). Ironically, the issue that will probably prove most problematic for the development of SPR-based protein-detecting arrays is that it may be difficult to carry out relative measurements of protein samples on the same array, which is what most users will want to do. Since SPR essentially responds to mass changes, there is no obvious analogue of a two-color label or isotopic labeling scheme that could be applied here. Therefore, one will likely have to attempt to contrast absolute signals obtained from two different spots, which has its own problems, including complications due to protein–protein interactions (see Section 6). Nonetheless, in the medium to long term, SPR is a promising technology in the protein array area, particularly when the field eventually turns to intrinsic quantitative measurements.

## 6. The complications of protein–protein interactions

As mentioned above, much of the thinking in the protein array area has been dominated by analogies with DNA microarrays. A particularly misleading facet of this mindset is that it leads workers in the field to ignore the consequences of the fact that proteins tend to associate

with one another. Indeed, it is now generally appreciated that most biological events are mediated by 'protein machines' [33] comprised of anywhere from several to almost 100 different polypeptides. This leads to a complication in the design of ligand discovery strategies that I believe is grossly underappreciated.

To obtain protein ligands, virtually everyone involved in this field plans to screen some sort of library (comprised of antibodies, peptides, nucleic acid or protein aptamers, or small molecules) against recombinant proteins or protein domains. However, the experience of several laboratories [34,35], including my own [28], is that the ligands obtained in such screens almost invariably bind to native interaction regions of the target protein. This is presumably because sites of native protein–protein interactions are the most 'bindable' regions of the protein. In other words, other surfaces of the protein have probably evolved to prevent non-specific interactions with other factors in the very concentrated milieu of the cell.

This has interesting consequences with regard to employing the isolated compounds as ligands for protein-detecting arrays. At least in experiments using cell extracts prepared under more or less physiological conditions, it seems likely that a majority of the ligands on the chip will have to compete with native factors for binding of the target protein. Depending on the relative affinities of the immobilized ligand and the native competitor protein for the target factor and several other factors (see below), this competition will result in only some fraction of the target protein in the extract binding to the chip (Fig. 6). Since the affinities of native protein–protein interactions can vary over several orders of magnitude, this means that the absolute amount of protein that binds to a spot on the array may diverge wildly from its true concentration in the sample. Some proteins may not bind at all if the ligand-binding site is occluded in the interior of a stable complex. This is one of many problems inherent in absolute measurements using this technology. The situation is analogous to one with which molecular biologists are quite familiar. If one isolates an antibody against a given protein, even if it performs well in a Western blot experiment where protein complexes have been disrupted, it may or may not be useful in an immunoprecipitation application. It all depends on the accessibility of the epitope recognized by the antibody.

The complicating effects of protein–protein interactions are ameliorated to some extent if one attempts only relative measurements between two samples. In other words, one would hope that the intrinsic 'partition ratio' between a target peptide binding to the immobilized ligand and the native competitor protein would be a constant one, therefore allowing comparative measurements between two different samples to be made. Unfortunately, this will not always be true. For instance, consider a case in which a change in the physiological state of the cell reduces the amount of competitor protein Y, which binds target protein X. Presumably, this would result in a significant increase in the amount of protein X bound to the appropriate spot on the array. Thus, it would be easy to misinterpret the data as indicative of that physiological stimulus resulting in a significant increase in the level of protein X. Of course, if one had an array that monitored every protein in the proteome, one might be able to deconvolute all of the data appropriately. But an array of

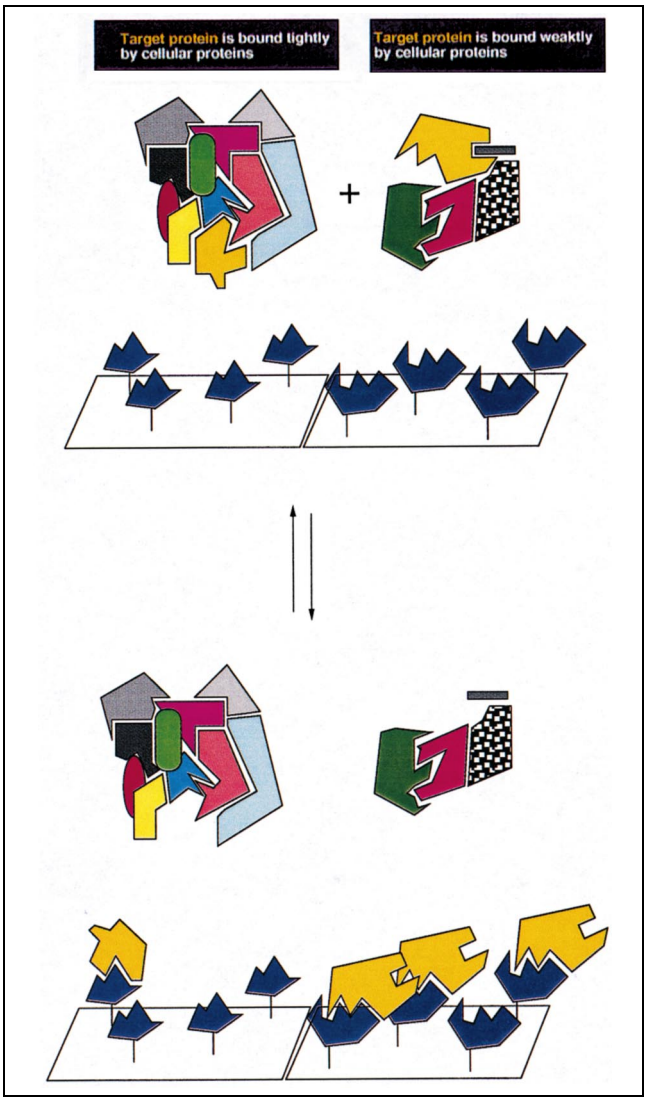


Fig. 6. The effect of protein–protein interactions on protein binding to immobilized capture ligands. This figure represents two multi-protein complexes, each containing a protein (orange) for which a specific ligand is present in the array (blue). The illustrations represent cases in which one protein is bound tightly in the complex, the other loosely. If the capture ligand must compete with the other proteins in the complex for the target factor, then the different affinities of the targets for their natural partners will result in different amounts of that factor being captured on the array. This simple analysis does not take into account the fact that the capture ligand–target protein affinities will undoubtedly also be different. Factors such as these will make absolute quantitation of protein levels using array technology extremely difficult. However, while other complications remain (see text), measurements of relative changes between two samples (Fig. 1, bottom) will be less troubled by such effects.

this complexity will not be available for some time and even then, such corrections would require an extensive knowledge of the network of protein–protein interactions in the proteome of interest and a complex bioinformatics approach to signal correction. Finally, the same problem could arise even if the level of protein Y does not change drastically. For example, suppose a change in conditions results in phosphorylation of protein Y and that this event drastically reduces its affinity for protein X.

The issue of protein–protein interactions provides special challenges to the development of SPR as a detection technique. Whether protein X binds to a given spot on a chip as a free polypeptide or as one of dozens of proteins in a stable complex will obviously provide grossly different signal intensities (Fig. 7). This is an advantage of MS over SPR as the most desirable 'direct analysis' tool for protein arrays, since MS will segregate all of the different proteins in the complex, allowing only the signal of protein X to be analyzed. There has been interest in coupling SPR with MS [36] and if the advantages of both techniques could be exploited, this might be a promising approach to detection.

A final point relevant to this topic is that it might be possible to avoid the problem if one could somehow demand that the ligand bind the target protein in a region that does not participate in native interactions. This will not be easy, but it may be possible. The simplest approach would be to employ as targets in the library screening experiments suitable peptide epitopes derived from the target protein rather than the intact protein itself. These epitopes would be selected based on structural knowledge or (more often) on bioinformatics-based guesses as to what regions of the protein are not likely to be involved in intermolecular interactions. Unfortunately, this approach will complicate ligand isolation, since it is going to be more difficult to obtain compounds that bind peptide epitopes than those that snuggle into the molecular crevices and caverns available in a folded protein. This is particularly true if one is talking about small molecule ligands. While one can imagine selecting monoclonal antibodies that bind peptide epitopes in a high-throughput fashion, the literature on epitope-binding small molecules is extremely slim. Indeed, to the best of my knowledge, there have only been two reports of screening libraries for chemically synthesizable molecules capable of binding peptide epitopes in aqueous solution, both involving epitope-binding peptides [37,38]. It will be interesting to see how this very nascent field develops over the next few years.

In summary, it is safe to say that the extensive networks of protein–protein interactions present in the cell will provide a considerable challenge to interpreting the data obtained from protein-detecting microarrays correctly. To be fair, it should be pointed out that this issue can probably be put off for a while, since there are applications for which it will not be a major issue. For instance, protein–protein interactions will be much less prevalent in blood samples than in cell extracts. Also, if one is looking for nothing more than a biosignature indicative of a disease state or a pathogen infection, then interpretation of the signal intensities is irrelevant. So clearly, this issue should not paralyze advances in the field. Almost every new technology has recognized pitfalls that are ignored in the first wave of development. However, for the core application of using protein-detecting arrays as a central tool in biological research, where cell extracts will likely be the most common sample, dealing with the impact of protein–protein interactions is an important problem that will eventually require considerable effort and imagination to solve.

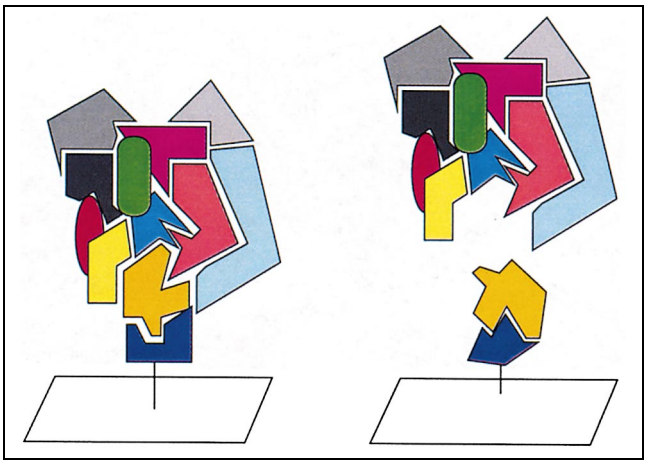

Fig. 7. Capture ligands that bind a surface of the target protein not occluded in native complexes (left) would not suffer from complications due to competing with native protein–protein interactions (right). However, since selection experiments using intact proteins or protein domains generally provide compounds that recognize native interaction surfaces, obtaining ligands such as that shown on the left may require using specific epitopes as targets in library screens. If SPR were employed to monitor binding, these two types of binding events would provide dramatically different responses. For this technique to be of use, therefore, one would have to know whether the capture ligand binds the target protein in isolation or as part of a complex. This is a significant obstacle to the implementation of SPR as an array analysis technique even if instruments capable of multiple measurements become available.

## 7. Ligands specific for a particular form of a protein

As discussed in Section 1, one of the most powerful arguments for the development of protein-detecting arrays is that DNA microarrays are blind to chemical alterations in the state of the protein that can have tremendous effects on its function. I used the term post-translational modification previously and, indeed, covalent modification of protein side chains or cleavage of main chains is one of the dominant mechanisms by which protein activity is regulated. However, it is important to point out that non-covalent events also can have important consequences on protein function. For example, the activity of Ras bound to GTP is very different than that of Ras com-

plexed to GDP. In general, therefore, one would ideally like to have ligands capable of distinguishing between different molecular states of a given protein in order to take full advantage of the potential of protein-detecting arrays.

This provides yet another challenge in the ligand screening area. The most straightforward approach would be to employ as a target in any given library screening experiment the desired form of the protein, for example phosphorylated at a given serine. But there is no guarantee that the ligands obtained in such an experiment would be specific for that form of the protein. Thus, some kind of rescreening against the unmodified form of the factor would be required. This may not sound so bad, but when extremely high-throughput is required, schemes requiring multiple subsequent steps after the initial screening event become cumbersome. Furthermore, there is no guarantee that any of the selected ligands will be specific for the desired form of the protein. Finally, in many cases it may be difficult to prepare in pure form the desired post-translationally modified version of the target protein. This is particularly true for phosphorylated proteins, where the kinase that operates on them is often unknown or not readily available.

One possible solution to this problem is to focus on epitopes as targets. If a 10–15 amino acid epitope containing a phosphorylated serine, for example, were employed as the target in a library screening experiment, it seems far more likely that the ligands isolated would distinguish between the phosphorylated or unphosphorylated forms. In addition, a phosphorylated peptide could be made synthetically, simplifying target preparation. Particularly in the case where phage-displayed single chain antibody libraries are employed as the source of protein-binding ligands, this may be the most effective strategy to obtain ligands with the desired specificity. To obtain relatively small molecules capable of distinguishing different forms of an epitope will be far more challenging.

## 8. Conclusion

Protein arrays will likely be the next major manifestation of the revolution in genomics and proteomics. An important distinction in this area is to recognize that two fundamentally different types of arrays will be developed. Protein function arrays, which will almost certainly come into general use first, will be comprised of native proteins arrayed on chips. These arrays will be extremely useful for studies of the activities and binding properties of native proteins and will also be of broad utility in addressing the specificity of protein-binding small molecules, including drug candidates. The second type of chip, which I have termed a protein-detecting array, will be used to monitor the levels and chemical states of native proteins. These arrays will be the proteomics version of DNA microarrays and will be utilized for proteome-wide profiling experiments. There are two major technical hurdles that must be addressed before relatively sophisticated protein-detecting arrays can be produced. One is the development of high-throughput technology for the isolation of high specificity and affinity protein ligands. The other is to devise a robust method to detect binding of the proteins in a biological sample to the array, preferably without the need to chemically modify the protein analytes. Once these issues are solved, there will remain a variety of interesting secondary challenges, including the development of methods to distinguish between different forms of the same protein and strategies to deal with the complications imposed by native protein–protein interactions. Therefore, it is safe to say that this will continue to be an interesting area in need of innovative thinking for several years.

It is difficult to overstate the importance of the development of practical devices of this sort. It would not be surprising if in 10–20 years time protein-detecting arrays form the backbone of medical diagnostics. This is an area of tremendous opportunity for biologically inclined organic chemists, particularly those with a strong background in physical chemistry and engineering (or with good collaborators in these areas). In particular, while the first generation protein-detecting chips will rely almost exclusively on antibodies or other macromolecules, there are many practical problems associated with making complex chips from these materials, particularly for mass production. It is my opinion that the full potential of this technology will be realized only when ligands that can be synthesized chemically become available for thousands of proteins. Indeed, while I have discussed the problems of ligand isolation and detection of binding as being more or less independent, this may not be the case. One can certainly imagine tailoring the ligands themselves to somehow aid in reporting when they bind their target protein. In considering such approaches, there is no substitute for the flexibility afforded by synthetic chemistry. Clearly, this is a problem made to order for chemical biologists. Indeed, it can be argued that the 'protein binding problem' is the ultimate challenge in molecular recognition today and one worthy of the attention of the best people in the field.

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