

MINIREVIEW

Emerging tools for real-time label-free detection of interactions on functional protein microarrays

Niroshan Ramachandran¹, Dale N. Larson², Peter R. H. Stark², Eugenie Hainsworth^{1,2} and Joshua LaBaer¹

¹ Harvard Institute of Proteomics, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Cambridge, MA, USA

² Technology & Engineering Center, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA

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Correspondence

J. LaBaer, Harvard Institute of Proteomics, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 320 Charles Street, Cambridge, MA 02141, USA

Fax: 617 324 0824

Tel: 617 324 0827

E-mail: josh@hms.harvard.edu

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The availability of extensive genomic information and content has spawned an era of high-throughput screening that is generating large sets of functional genomic data. In particular, the need to understand the biochemical wiring within a cell has introduced novel approaches to map the intricate networks of biological interactions arising from the interactions of proteins. The current technologies for assaying protein interactions – yeast two-hybrid and immunoprecipitation with mass spectrometric detection – have met with considerable success. However, the parallel use of these approaches has identified only a small fraction of physiologically relevant interactions among proteins, neglecting all nonprotein interactions, such as with metabolites, lipids, DNA and small molecules. This highlights the need for further development of proteome scale technologies that enable the study of protein function. Here we discuss recent advances in high-throughput technologies for displaying proteins on functional protein microarrays and the real-time label-free detection of interactions using probes of the local index of refraction, carbon nanotubes and nanowires, or microelectromechanical systems cantilevers. The combination of these technologies will facilitate the large-scale study of protein interactions with proteins as well as with other biomolecules.

The wide variety of protein interactions in a cell comprises a biochemical wiring network that controls everything from growth and division to the cell's response to its environment. These interactions include metabolites, lipids, nucleic acids, carbohydrates, proteins (both self and other proteins) and drugs [1–7]. Understanding the dynamic nature of these interactions will reveal the functional responsibilities of proteins and the circuits in which they operate [8]. The

complex milieu of the living cell has slowed many attempts at assaying for protein function *in vivo*. The broad dynamic range of protein abundance in biological samples [9,10], and the ability of proteins to undergo post-translational modifications (PTMs), such as phosphorylation, glycosylation and myristoylation, further encumber the ability to build sensitive and accurate assays for studying protein function. This is a result of the dependence of many protein interactions

Abbreviations

ASR, analyte-specific reagent; GC-SPR, grating-coupled surface plasmon resonance; HT, high-throughput; IP, immunoprecipitation; IP/MS, immunoprecipitation with mass spectrometric detection; MEMS, microelectromechanical systems; NAPPA, nucleic acid programmable protein array; PTM, post-translational modification; RIU, refractive index units; SPR, surface plasmon resonance; YTH, yeast two-hybrid; μ SERS, micro surface-enhanced Raman scattering.

on protein levels and the presence of one or more PTMs [9–11]. A detailed understanding of protein interactions, including their kinetics, affinities, and factors such as pH, ionic strength, and temperature, which affect the thermodynamics, will provide the best possible opportunity to develop wiring diagrams that correctly model protein functional behavior [12].

The recent development of functional protein microarrays, on which thousands of discrete proteins are printed at high spatial density, offers a novel tool for using to interrogate protein function in high-throughput (HT). Until recently, these microarrays relied on some form of labeling on the query molecule used to probe the target proteins on the arrays. The emergence of sensitive real-time label-free detection systems that use probes of local index of refraction [e.g. surface plasmon resonance (SPR) methods], carbon nanowires, nanotubes, and microcantilevers may provide crucial tools that are needed to empower the use of protein microarrays in experiments previously unattainable. Here we will review the development of functional protein microarrays and promising technologies for the real-time label-free detection and characterization of protein interactions that may provide higher resolution functional data.

Common methods for studying protein interactions

Current approaches for studying protein interactions include solution biochemistry using purified proteins, immunoprecipitations (IP) or tagged-based affinity purifications [e.g. tandem affinity purifications (TAP)] and the yeast two-hybrid (YTH) system. Traditional biochemical methods in which proteins are purified and their activities probed in solution often provide high-resolution data regarding the kinetics and thermodynamics of the interactions. However, this approach has not been extended to whole proteome studies, and hence is not reviewed here. With IP, the protein of interest is isolated from a complex mixture, such as a cell lysate, using an analyte-specific reagent (ASR; e.g. antibody) along with its interacting partners (Fig. 1A). Alternatively, the protein of interest can be fused to a high affinity tag and isolated from the complex mixture using the appropriate capture reagent. The use of IP allows endogenous proteins to be isolated without the need for cDNAs encoding the protein of interest or the need to express the fusion construct; however, for HT applications this would require a specific ASR for every protein of interest, whereas tag-based affinity purifications using a single isolation chemistry can be applied to all proteins. To identify the binding part-

ners, the proteins that associate with the index protein are often separated by gel electrophoresis and can be probed either with specific reagents on western blots (if their identities are suspected and the corresponding reagents are available), or they can be digested before or after separation by specific proteases followed by analysis on a mass spectrometer [13,14]. This approach has the potential to capture natural protein complexes and does not require that the interacting proteins are known in advance or that their genes are even cloned. However, because all the proteins co-purify together, this method cannot determine which proteins are in direct contact with one another.

In contrast to direct biochemistry and IP-based methods, which are primarily *in vitro* biochemical methods, the YTH detects interactions *in vivo* in yeast cells. This is accomplished by measuring the signal from reporter genes whose transcription is induced when their cognate transcription factors are reconstituted by bringing together two functional halves through the interaction of the linked proteins [15]. A variety of reporter systems have been adapted to the YTH system, usually expressing enzymes that either produce metabolites to support growth or induce color changes in specific substrates. The YTH systems specifically measure binary interactions, although the interactions must occur in yeast, and specifically in the context of the yeast nucleus, which may lead to false negatives, especially for some mammalian proteins. To address this, similar approaches, called mammalian two-hybrid systems, have been developed that reconstitute active domains of reporter proteins, such as functional enzymes, ubiquitin, fluorescent proteins, and others to demonstrate the presence of an interaction [16–21]. Mammalian two-hybrid systems have been successfully used to monitor protein interactions in the cytosol as well as among membrane-bound proteins, but challenges associated with the HT introduction of DNA into mammalian cells, and the need for high-quality libraries of genes to test, has limited their widespread adoption [22].

Adapting current interaction methods for HT experiments

Both the YTH and IP methods have been used to map protein–protein interactions at the proteome scale in several organisms [23–28]. Several large scale efforts using either IP coupled to mass spectrometric detection (IP/MS) or the YTH system have been used to identify protein interactions in the yeast proteome. These efforts revealed large convoluted protein interaction networks, illustrating the complex behavior of proteins

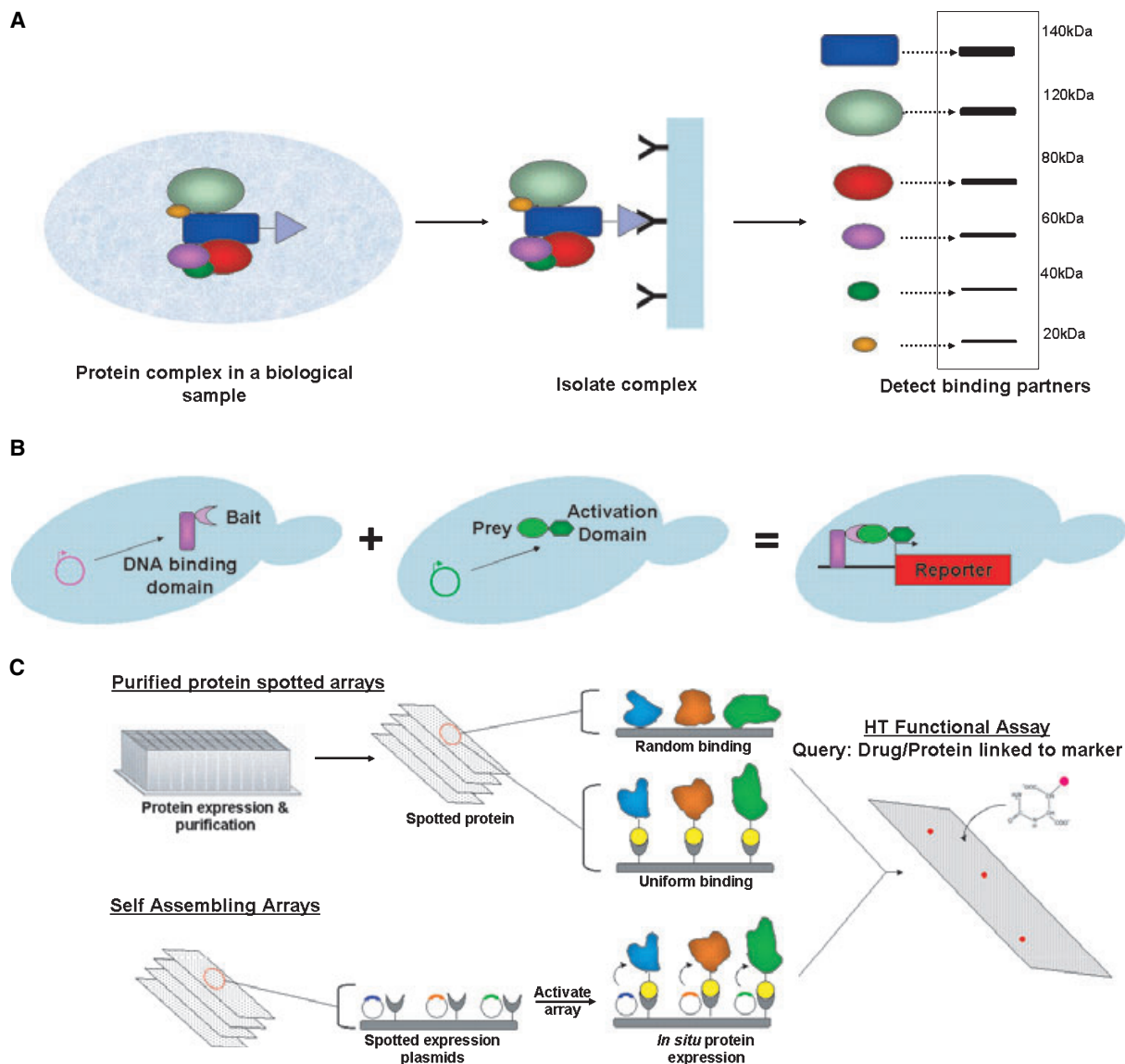


Fig. 1. (A) Immunoprecipitation. Proteins of interest (rectangle) can be isolated from complex biological sample by using antibodies specific to the protein or by modifying the protein with a tag (triangle). The protein of interest and its binding partners can then be separated based on charge, size or isoelectric point, and detected using antibodies specific to the interacting partners or by using mass spectrometry. (B) Yeast two-hybrid. A cell is programmed to express a bait protein, which is fused to a DNA-binding domain and mated with another cell expressing the prey fused to the activation domain. The DNA-binding domain and the activation domain are necessary to bind to the promoter element and recruit transcription factors necessary for gene expression. Here, the interaction between the bait and prey brings together the factors necessary to activate the expression of the reporter gene. (C) Functional protein microarrays. Top: purified protein spotted array. Proteins are expressed and purified in high throughput and spotted onto a solid surface. Proteins are bound in a random orientation or uniform orientation by modifying the N or C terminus of the protein with a capture tag. Bottom: self-assembling array. Arrays can be programmed with cDNAs for *in situ* expression of the desired proteins. Proteins are expressed using a mammalian cell-free expression system and immobilized in a uniform orientation using a C-terminus capture tag. Both arrays can be probed with labeled query, and the binding can be detected using fluorescence microarray readers.

[29–31]. Amidst these data were interactions that were biologically relevant and some that appear to be artifactual products of the assay. One striking observation was that comparable efforts from multiple laboratories

using either the YTH system or IP/MS revealed only a 10% overlap in the number of interactions identified in yeast, regardless of the method used and despite testing similar gene sets [30]. This lack of concordance

suggests a large false negative rate, and a potentially large false positive rate for these methods. Thus, although these methods are well established, yield valuable data and adapt well to proteome scale application, there is room for orthogonal HT protein interaction detection technologies that will help to validate interactions detected by these methods and to identify novel interactions. Furthermore, a significant limitation of all these methods is that they are limited to detecting only protein–protein interactions. Methods that could also detect interactions between proteins and other biomolecules, such as lipids, nucleic acids, and small molecules in HT, are sorely needed.

Protein microarrays: introduction

The use of protein microarrays to study the biochemistry of proteins offers advantages over the currently used technologies (Fig. 1C) [32–36]. Compared with solution biochemistry, thousands of different proteins can be interrogated using very small sample volumes, and compared with YTH and IP approaches, interactions with other biomolecules can also be assessed. For example, an array of proteins can be probed with fragments of DNA, corresponding to promoter regions of the genome, to identify DNA-binding proteins, or a family of proteins (such as proteases) can be screened with a small drug molecule to identify potential inhibitors of selective proteases [37,38]. Protein microarrays can be used to identify substrates for post-translational modification by screening the target proteins on the array with modifying enzymes, such as kinases, combined with a detectable substrate (e.g. radioactively labeled ATP) [39]. This strategy provides functional information regarding the specificity of modifying enzymes, as well as the suitability of a large number of proteins as substrates. Moreover, in contrast to the YTH system, where interactions must occur in the nucleus, the open format of functional protein microarrays allows greater flexibility to manipulate the assay parameters. For example, query molecules can either be introduced as purified proteins or presented mixed with various biological samples, such as cell lysates, tissue extracts or serum [40].

Protein microarrays

Challenges

Compared with DNA microarrays, building functional protein microarrays adds several major challenges. First, whereas short nucleotide sequences (≈ 20 bases) are sufficient to provide the necessary gene-specific

information needed for DNA arrays, the full-length coding sequence is required to obtain functional protein. The protein coding sequence can vary from a few hundred bases to over 10 000 bases, which demands that protein production for protein microarrays should be robust over a large dynamic range. Second, methods to amplify nucleic acids for printing have become routine, with both enzymatic and chemical methods available, whereas protein microarrays require robust HT methods to express and purify proteins, with good yield, that retain natural folding [41,42]. To achieve this, it would be ideal to express proteins in a homologous system; however, this can be difficult for mammalian proteins. The third challenge is to immobilize the proteins without altering their native functional state. Regardless of their specific sequence, all nucleic acids share a common chemistry that can be exploited in affixing them to DNA arrays, but the staggering diversity of chemistries for proteins makes it more challenging to find a single chemistry that can efficiently immobilize all proteins without affecting function. Finally, it is important that the immobilization chemistry provides access to all surfaces of the protein.

The production of target proteins for protein microarrays relies on the availability of large cDNA collections and methods to produce proteins in HT. The cDNA collection must be in an expression-ready format, without untranslated sequences, and with the coding sequences linked to the appropriate promoter and necessary purification tags. The increased availability of cDNA collections built in recombinational cloning vectors simplifies the transfer of coding sequences into protein expression-ready formats [41–46]. Once transferred, however, producing functional protein in HT still remains a challenge. Current methods commonly rely on bacterial systems, where 60% of the mammalian proteins are expected to be expressed [41]. The concern is whether the quality of proteins from these HT approaches will be sufficient for functional assays.

The capture of proteins to an array surface is challenging, given the complexity of their chemistry and the need to maintain their integrity and accessibility on the array surface. Currently, there are two approaches for protein capture to the array surface: random and uniform [47,48]. Proteins can be immobilized onto the surface in a random orientation using aldehyde, epoxy, amine or other chemistries that react to amine and carboxy groups of the protein, allowing the protein to bind in a number of different orientations [32]. This approach ensures that many faces of the protein are exposed for potential interactions, although it tends to hold proteins close to the array surface. Alternatively, proteins can be tagged at the N or C termini and

immobilized via the tag to the surface, which is coated with a corresponding capture agent, ensuring that all proteins are oriented uniformly. This has the advantage that proteins are held away from the surface, minimizing steric hindrance. The tag also provides an added level of selectivity for the binding of the protein of interest, so that protein purification does not have to be as extensive. To date, both approaches have proved adequate for assaying protein function [48].

As many proteins are labile, the entire process of expression, purification, spotting, and microarray storage conditions must be conducive to maintaining protein integrity. Inactive or denatured proteins contribute to false negatives as they may fail to function during assays, and false positives may also occur owing to artifactual interactions with ordinarily cryptic sites exposed by denaturation. Moreover, the denaturation of proteins on the arrays will occur sporadically, affecting some proteins more than others, making it difficult to know which proteins retain function. There are no useful tests that can be employed on microarrays to confirm proper folding for all proteins. Thus, it is best to minimize the manipulation of the proteins and to produce them as close as possible to the time of assaying. A useful strategy in this regard is the self-assembling protein microarray, called nucleic acid programmable protein array (NAPPA), which reduces the process of building protein microarrays to a single step (Fig. 1C; bottom) [49]. This approach entails the spotting of expression plasmids, instead of purified proteins, on the array surface and using a mammalian cell-free expression system to express the proteins *in situ* at the time of the assay. All proteins are expressed with fusion tags that correspond to capture agents printed along with the plasmid DNA and act to capture the protein as soon as it is translated. This chemistry expresses and captures almost 1000-fold more protein per spot than conventional protein spot arrays [48]. By producing the proteins just-in-time for assay, the opportunity to denature is significantly reduced, and the use of a mammalian transcription/translation system encourages natural protein folding for mammalian proteins. Early applications of this approach show promise, although it is too early for significant experience to have accrued.

Applications

The challenges facing protein microarrays are yielding to various successful efforts to build the arrays, and they have now been used successfully to study protein function through detecting protein–protein interactions; protein interactions with small molecules, lipids, nucleic

acids, antibodies; and in screening experiments for enzyme substrates [32,38,47,48,50–53]. Protein microarrays can also be used to display variants or deletions of a single protein. For example, Boutell *et al.* generated an array of p53 variants to study the effects of mutations and polymorphisms on the ability of p53 to bind the GADD45 promoter element, interact with the MDM2 oncoprotein, and serve as a substrate for phosphorylation by casein kinase II [37]. This type of study can help to elucidate the functional roles of specific proteins in the pathophysiology of diseases such as cancer.

Protein microarrays appear to be very efficient at detecting protein–protein interactions. In one experiment, each member of 30 human DNA replication proteins was used to probe an array of the entire set to interrogate all of the 900 possible binary interactions [49]. Of these, 110 interactions were detected, including 40 interactions that were previously unreported. The ability to detect 85% of the interactions previously identified using biochemical methods corresponds to a very low false negative rate and confirms the functional integrity of the proteins on the array. In addition to detecting binary interactions, this approach was used to build multicomponent systems, as well as to identify the interacting domains of proteins.

Identifying the functions of protein domains may help to assign function to novel proteins based on their domain composition. In many cases the protein interactions are driven by specific domains, and therefore it is important to identify interactions among the building blocks of the protein [4,5,54]. Espejo *et al.* characterized the function of protein domains by purifying and displaying over 200 protein fragments, of which 145 were known protein domains (PDZ, SH2, SH3 and others), and probed them with biotinylated peptides [40]. The peptides encoding different motifs (P3, PPYP, PGM) bound specifically to their respective domains, demonstrating that the immobilized domains were functional. Methylation of peptides altered their binding profile, revealing the effects of PTMs on the specificity of binding. Larger scale application of this approach promises to generate useful binding profiles for proteins, domains and their modified forms [40].

The detection of signal for all current HT protein interaction technologies at some level require either that an ASR is available for the query molecule or that the query molecule is somehow labeled (e.g. radioactively, fluorescent dye, epitope tag). Existing collections of ASRs cover only a very limited fraction of the proteome and are unlikely to be available for most metabolites or drugs. Labeling query molecules for large scale studies can often be tedious, expensive and

not easily generalizable. Depending on the size and position of the label, it may affect the query molecule's ability to interact with the target proteins either because of conformational strains on the protein or steric hindrance. The effect is more likely to be pronounced when labeling query molecules that are smaller than proteins, such as metabolites, oligonucleotides, peptides, and especially small organic molecules. Thus, the ability to map protein interactions with nonprotein biomolecules will depend on the development of label-free methods for measuring the interactions. Coupling functional protein microarrays to real-time label-free detection systems would enable a paradigm shift in our ability to understand protein interactions with biomolecules at the proteome scale.

Real-time label-free detection

The key requirements for any new label-free protein microarray sensing technology are that it should be compatible with HT (multiplexed detection) methods, should be able to detect small molecules binding to immobilized protein targets, should be able to detect interactions involving biomolecules present at low concentrations in the sample, and have a wide dynamic range. In addition, if the sensor will be used to measure binding kinetics, it needs a sufficiently high sampling frequency to capture the shape of the binding curve.

The performance of a sensing technology is often characterized by sensitivity, resolution, and detection limit. Sensitivity is the derivative of the measured parameter with respect to the parameter to be determined. In the case of fluorescence detection and protein arrays, the measured parameter is fluorescence intensity and the parameter to be determined is the number of molecules bound to the immobilized protein. Resolution is the smallest change above the noise floor of the detector in the measured parameter that can be reliably detected. These are critical parameters because their values indicate the feasibility of using a technology for a specific experiment. For example, when studying small molecule interactions with immobilized proteins, the resolution governs the smallest molecular weight for the small molecules that can be studied. Sensitivity and detection limit will govern the lowest concentration of an analyte that can be detected.

Challenges with label-free detection

In experiments where a complex sample is being studied using a label-free detection technology, the issues of specificity and false positives owing to nonspecific binding can become a concern. There are two primary

sources of nonspecific binding for protein arrays: adsorption to the sensor surface and nonspecific binding to the immobilized proteins. Nonspecific binding to the sensing surface is usually addressed by designing a bioresistant surface chemistry. Although traditional microarray surface chemistries are based on derivatized glass surfaces that are susceptible to a higher degree of nonspecific binding, most label-free systems rely on gold-coated surfaces, which can be treated to have low nonspecific binding [55]. The nonspecific binding to the immobilized protein relies on the inherent reactivity of the query and the target protein; this is also an issue for YTH and IP. Non-specific binding is sometimes addressed by assaying at higher stringencies, but this will detect only the strong and stable interactions, not the weak and transient interactions. Thus, nonspecific binding continues to be an issue for most assay systems.

Current technologies

Conventional SPR has become the technology of choice for label-free detection studying binding kinetics [56], but the level of multiplexing that has been shown is limited to 50 [57,58] to 64 [59] spots. Fortunately, there are several technologies at various stages of development that have the promise to meet the sensing needs of protein microarrays. These technologies include: several different technologies that probe the local index of refraction (one of which is conventional SPR); carbon nanotubes and nanowires; and microelectromechanical systems (MEMS) cantilevers. Other technologies, such as Kelvin nanoprobe [60,61], micro surface-enhanced Raman scattering (μ SERS) [62], liquid crystal sensors [63], microsphere cavities [64], calorimetry using enthalpy arrays [65], and several interference methods, including ellipsometry [66,67], interferometry [68–71] and reflectometric interference spectroscopy [72,73], are in various stages of development and may have an impact, but are not reviewed here.

In addition to the sensing technologies, improved technology infrastructure will need to be developed. Infrastructure items include: technology-specific instrumentation; technology-specific assays and methods; the ability to spot arrays at very high spatial density; and informatics approaches to analyze the data.

Probes of the local index of refraction

Changes in the local index of refraction [74–77] can be monitored to detect and characterize binding interactions between a query molecule and immobilized

protein. These changes in the local index of refraction alter a plasma wave established in the metallic surface [78] of the sensor and are measured optically. For SPR [77], the monitored optical parameter can be: the angle at which photons resonantly couple with free (valence) electrons in the metallic surface of the sensor; the wavelength where resonant coupling occurs; intensity; the phase of the light; or modulation of the light's polarization. Conventional SPR [79,80] typically measures shifts in the angle at which resonant coupling takes place, (Fig. 2A). Commercially available SPR systems have flowcells that provide a small degree of multiplexing with independent channels for different immobilized proteins (4 [56] to 25 (GWC Technologies, www.gwctechnologies.com)) and a research instrument has achieved multiplexing with 50 [57,58] to 64 [59] spots. There are three technologies in development that are intended to extend local index of refraction probes into multiplexed detection. These technologies are grating-coupled SPR (GC-SPR), colorimetric resonant reflection, and nanohole array sensors.

For all of these technologies, the magnitude of the change in the local index of refraction caused by binding is a function of the mass and conformation of both the query molecule and the immobilized protein, the number of query molecules that are bound to the immobilized protein, and the distance of the bound query molecule from the sensing surface. A summary of their detection performance is shown below, in Table 1. The resolution of these technologies is compared in refractive index units (RIU) which has little biological significance, but does allow quantitative comparisons.

Conventional SPR has been used extensively to study binding interactions with proteins [56,83], including the interactions between proteins and small organic molecules, peptides, nucleic acids, and protein drugs. The detection principle is shown in Fig. 2A. It has been used to provide concentration information, as well as data on binding kinetics. However, current SPR technologies are not highly multiplexed [57,58].

GC-SPR [77,81,90] monitors changes in reflectivity, and an area detector (e.g. CCD camera) is used to record the reflectance from different locations on the sensor surface, but at the expense of sensitivity [81–83]. The detection principle is illustrated in Fig. 2B. The ability to have 400 independent assays on a sensor chip has been shown with this technology. GC-SPR has enough similarity to conventional SPR to ensure that all of the assays which have been performed using conventional SPR will migrate to GC-SPR platforms, with the exception of those where the sensitivity of

GC-SPR is inadequate. This technology is at the prototype stage of development.

Colorimetric resonant reflection [85,91,92] detects binding by measuring changes in the wavelength of light reflected from a subwavelength grating structure that has been appropriately functionalized (Fig. 2C). Colorimetric resonant reflection has been used in a 96-well format to detect protein–protein interactions, protein–small molecule interactions and even the cleavage of a portion of a bound molecule [85,88,93].

Nanohole array sensors measure changes in the amount of light transmitted through 150 nm diameter nanoholes [89] or through shifts in the emission spectrum of light emitted from 200 nm nanoholes [94]. This technology is shown in Fig. 2D. In 1998, Ebbesen and colleagues [95–100] demonstrated extraordinary optical transmission through nanoscale apertures that was several orders of magnitude greater than predicted by conventional optical theory. Changes in the ability of the nanoholes to transmit light are directly related to the local index of refraction of the sensing surface and are used as the basis for a new sensing technique. This coupling method allows for an individual sensor to be as small as $0.045 \mu\text{m}^2$, which is more than two orders of magnitude smaller than the theoretical limit for conventional SPR, with no compromise in sensitivity. This small sensor size enables the fabrication of a large number of independent sensors in a given area and very low reagent usage. The small sensor area also permits the analysis of very small samples, including tissue biopsies and cells collected by laser capture microdissection. This technology has demonstrated resolution of 9.4×10^{-8} RIU [89], which exceeds all other local index of refraction technologies. A proof-of-principle experiment, detecting the binding of glutathione-S-transferase (GST) to immobilized anti-GST, showed that GST at a concentration of 500 pM, binding to immobilized anti-GST, was easily detected (A. Halleck, P. Stark, D. Larson, unpublished results).

Carbon nanotubes and nanowires

Carbon nanowires represent an early stage technology that has the potential to address the needs of label-free sensing for protein arrays. The nanowires are functionalized and the conductance of the nanowire changes as the target molecules bind to the functionalized nanowires [101]. The detection principle is shown in Fig. 3. This sensing technology has been used to detect the binding of single virus particles [102], small molecule binding to proteins (1 nM Gleevec in the presence of 100 nM ATP binding to Abelson murine leukemia viral oncogene homolog (ABL); and 100 pM ATP binding

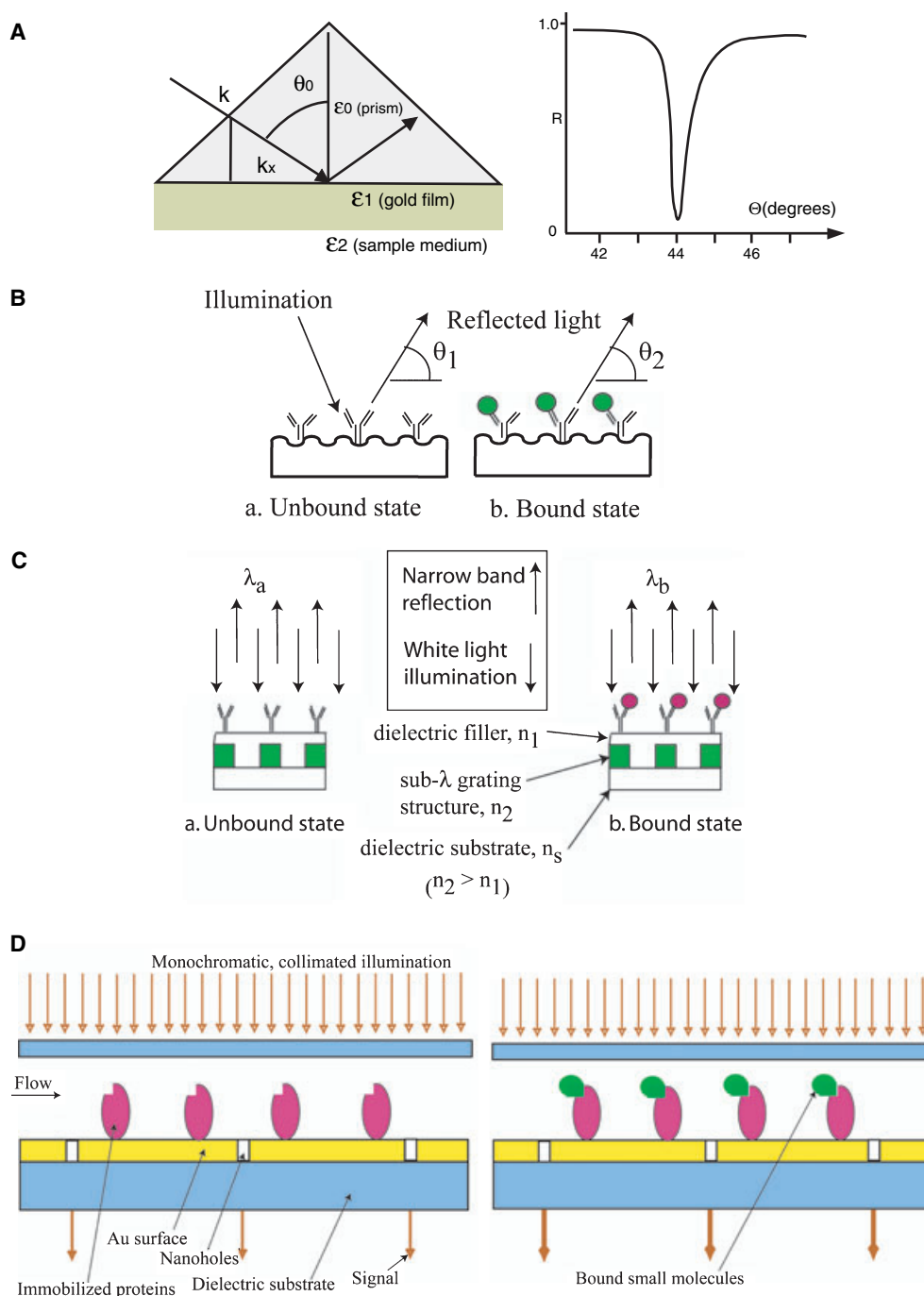
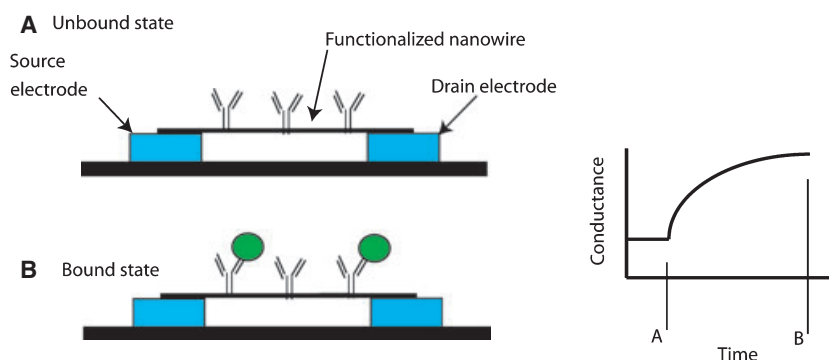


Fig. 2. (A) Local index of refraction. Surface plasmon resonance angular detection. Binding events are monitored via shifts in the angle at which resonance (as indicated by a large reduction in reflectance) occurs. k , Wavevector; ϵ , dielectric function; θ , angle of incidence of the light; R , reflectance. (B) Grating-coupled surface plasmon resonance (GC-SPR) achieves photon to plasmon coupling through grating momentum. GC-SPR has the same detection options as conventional SPR. Angular detection is shown in this figure. (C) Colorimetric resonant reflection measures changes in the reflected wavelength ($\lambda_a - \lambda_b$) caused by binding to the functionalized surface of the sensor. n , Index of refraction. (D) Nanohole array. Partial cross-section of a nanohole array sensor, showing detection of small molecules binding to protein targets immobilized on the nanohole array sensor surface. The intensity of the light emitted from the nanometric apertures changes as a result of the binding events.

Table 1. Summary of detection properties for leading biosensor technologies. RIU, refractive index units; SPR, surface plasmon resonance.

Technology	Resolution (RIU)	Multiplexing
Conventional SPR [57–59,81]	1×10^{-7} (angular interrogation) 2×10^{-5} (wavelength interrogation)	50 (using wavelength interrogation) or 64 (using reflectance intensity interrogation)
Grating-coupled SPR [82–84]	2×10^{-6} (8 kDa minimum size)	≈ 400
Colorimetric resonant reflection [84–88]	3.4×10^{-5} (308 Da demonstrated)	100 proteins per well in a 96-well format
Nanohole arrays [89]	9.4×10^{-8}	> 1 million spots per square mm is possible

**Fig. 3.** Carbon nanowire sensors show a change in conductance as proteins bind to a functionalized nanowire that bridges between two electrodes.

to ABL) [103], streptavidin (at 10 pM) binding to biotin [101] and anti-biotin immunoglobulin (at ≈ 4 nM) binding to immobilized biotin [101]. Carbon nanowires have also been used to detect nucleic acid hybridization [104] ($< \approx 1000$ copies bound in a $20 \mu\text{m} \times 20 \mu\text{m}$ area) and to detect single-strand binding proteins binding to DNA [105] (resolution of $0.15 \mu\text{g}\cdot\text{mL}^{-1}$ target DNA). Wang [103] *et al.* claim that nanowire sensors have advantages over conventional SPR in the areas of sensitivity, smaller quantity of protein needed for analysis, and the potential for very large arrays. The potential for small sample volume and large arrays can be attributed to the sensor size, which can have a mean wire diameter from 30–100 nm [104,106], and lengths from ≈ 5 –10 μm [104], and sensor spacings between 50 nm and 2 μm [103,106]. Arrays of nanowire sensors with 2400 independent sensors have been fabricated [106,107]. This technology makes use of well established electrical detection methods with excellent sensitivity and sampling rates.

MEMS cantilever sensors

Microcantilevers for biosensing are silicon strips of material attached at one end ('diving boards'), with a capture molecule, such as an antibody or a protein, bound to one surface. An analyte binding to the microcantilever is detected by measuring the bending of the cantilever as a result of surface stress, or by

measuring a change in the mechanical resonant frequency of the cantilever (Fig. 4).

For microcantilevers that detect bending, the analyte is allowed to bind to one side of the cantilever, either by functionalizing only one side for binding or by exposing only one side to the analyte. Binding produces either a tensile or a compressive stress at the surface, causing the cantilever to bend. The bending can be detected by the deflection of an optical beam [108–111], or by a change of electrical resistance in a piezoelectric thin film on the cantilever [112]. For microcantilevers that detect changes in resonant frequency, the piezoelectric thin film approach allows electrical excitation of the cantilevers and detection of their vibration [113]. While biological experiments to date have used small numbers of cantilevers, arrays of over 1000 cantilevers have been fabricated [114].

This technology has been successfully used for several applications. Fritz *et al.* demonstrated the ability to distinguish a single-base mismatch in the hybridization of two 12-mer DNA oligomers [115]. Using immobilized specific antibodies, Wu *et al.* demonstrated detection of prostate-specific antigen in its free (fPSA) and complexed (cPSA) forms, with slightly better sensitivity for cPSA. With a longer cantilever (600 μm rather than 200 μm) they were able to detect fPSA at $0.2 \text{ ng}\cdot\text{mL}^{-1}$ in a background of $1 \text{ mg}\cdot\text{mL}^{-1}$ BSA [109]. Savran *et al.* showed the binding of *Taq* polymerase to an immobilized anti-*Taq* aptamer. By running various concentrations from 0.3 to 500 pM, they

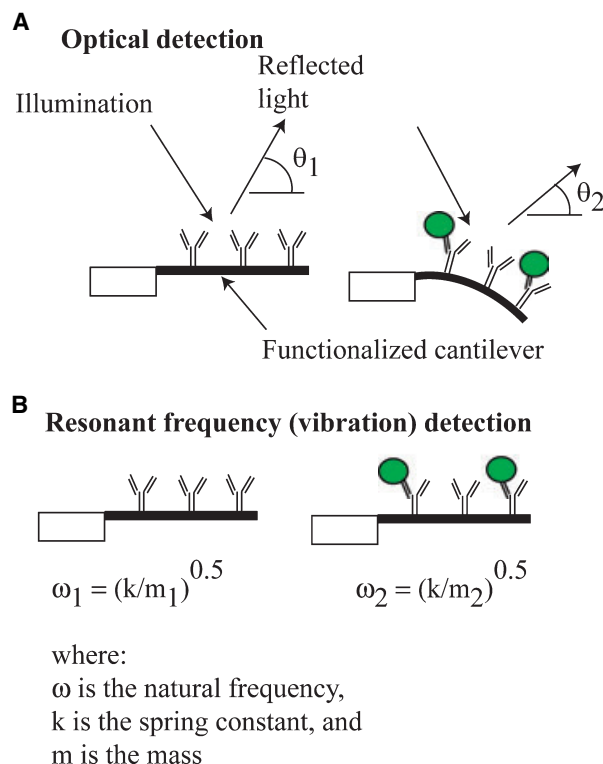


Fig. 4. Microelectromechanical systems (MEMS) cantilever sensors. A single cantilever is depicted. Protein molecules binding to antibodies cause the cantilever to bend. In this illustration, the bending is detected optically by the displacement of a beam of light reflected off the cantilever. Alternatively, the binding of the protein molecules changes the mass of the cantilever, altering the cantilever's resonant vibrational frequency, which is sensed electronically. θ , Angle of the reflected light.

determined a K_d of ≈ 15 pM, and they demonstrated detection of 50 pM *Taq* in a solution containing $18.5 \text{ ng} \cdot \text{mL}^{-1}$ of cell lysate [111].

Conclusions

Protein microarrays are powerful tools for large-scale biochemical analysis of protein function. However, widespread implementation of this technology has been limited owing to the cost and effort associated with producing thousands of proteins associated with assembling the arrays and the need to modify the query molecule in order to detect it. The available content for protein microarrays is accruing with increasing collections of genes in expression-ready format. A novel approach involving *in situ* expression of protein from immobilized cDNAs avoids the need for purification and allows for rapid production of proteins on the array. The need for labeling query molecules for detection has limited the number and types of mole-

cules tested, eliminating most nonprotein analytes. Real-time label-free technologies offer a way to avoid this limitation, and in addition, provide kinetic data. The leading technology in the field of label-free detection of biomolecular interactions is conventional SPR; however, in its current configuration, it does not have sufficient multiplexing capabilities to match the demands of today's protein microarray technology. The adoption and use of GC-SPR and colorimetric resonant reflection technologies is expected to be enhanced by the experience that has been gained using conventional SPR. Carbon nanowires/nanotubes and nanohole array sensors have potential as next-generation technologies, offering excellent sensitivity and high levels of multiplexing. As these technologies are developed and become available, their use in protein microarrays is expected to become routine. The ability to monitor the interactions of thousands of proteins in parallel and in real time has tremendous implications in the area of functional and clinical proteomics.

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