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Protein microarrays and novel detection platforms

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The field of proteomics has undergone rapid advancements over the last decade and protein microarrays have emerged as a promising technological platform for the challenging task of studying complex proteomes. This gel-free approach has found an increasing number of applications due to its ability to rapidly and efficiently study thousands of proteins simultaneously. Different protein microarrays, including capture arrays, reverse-phase arrays, tissue microarrays, lectin microarrays and cell-free expression microarrays, have emerged, which have demonstrated numerous applications for proteomics studies including biomarker discovery, protein interaction studies, enzyme–substrate profiling, immunological profiling and vaccine development, among many others. The need to detect extremely low-abundance proteins in complex mixtures has provided motivation for the development of sensitive, real-time and multiplexed detection platforms. Conventional label-based approaches like fluorescence, chemiluminescence and use of radioactive isotopes have witnessed substantial advancements, with techniques like quantum dots, gold nanoparticles, dye-doped nanoparticles and several bead-based methods now being employed for protein microarray studies. In order to overcome the limitations posed by label-based technologies, several label-free approaches like surface plasmon resonance, carbon nanotubes and nanowires, and microcantilevers, among others, have also advanced in recent years, and these methods detect the query molecule itself. The scope of this article is to outline the protein microarray techniques that are currently being used for analytical and function-based proteomics and to provide a detailed analysis of the key technological advances and applications of various detection systems that are commonly used with microarrays.

KEYWORDS: gold nanoparticles • label-based detection • label-free detection • microcantilevers • protein microarrays • quantum dots • SELDI-TOF • surface plasmon resonance

The study of the proteome, which encompasses the entire protein complement expressed by the genome of an organism at a given time, can be a daunting task due to the extremely large number of proteins present. The classical proteomics approach, which utilizes gel-based 2D electrophoresis and analytical mass spectrometry (MS) techniques, presents an important platform for the analysis and identification of proteins [1]. However, owing to many inherent drawbacks associated with gel-based methods, including lack of reproducibility and high-throughput (HT) analysis, numerous gel-free protein microarray techniques have been developed in recent years that carry out rapid functional analysis with economic use of samples and reagents. Protein microarrays are miniaturized 2D arrays, typically printed on functionalized glass slides, which consist of thousands of immobilized proteins that can be simultaneously studied and analyzed in a HT manner. Several microarray formats like capture arrays, reverse-phase arrays, tissue microarrays,

small-molecule microarrays [2,3] and microarrays generated by cell-based and cell-free techniques [4] have emerged over the years in order to suit the needs of various applications (FIGURE 1). These microarrays provide a versatile platform for many diverse applications, such as biomarker identification, protein interaction studies, immunological profiling and vaccine development, among others.

Along with the advancement of microarray technologies, the development of sensitive and reliable detection systems has also been imperative. Sophisticated label-based and label-free detection techniques have successfully kept pace with the increasing demands of protein microarrays in studying large numbers of proteins, their interactions and functions (FIGURE 1). Label-based systems involve the use of a tag for the query molecule in the form of conventional fluorescent dyes and radioisotopes, among others [5,6], or more recently, substances like inorganic quantum dots (QDs), gold nanoparticles (NPs), Raman dye-labeled carbon nanotubes [7]

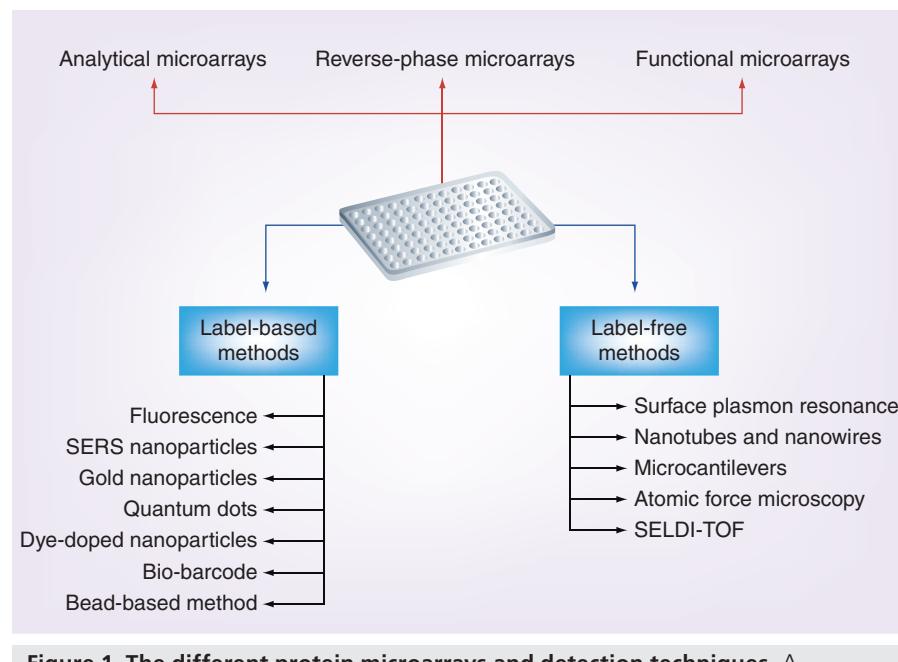


Figure 1. The different protein microarrays and detection techniques. A depiction of the various commonly employed formats for constructing protein microarrays based on the two broad categories of analytical and function-based arrays. The label-based and label-free detection techniques that can be used for protein microarray studies have also been depicted.

SERS: Surface-enhanced Raman scattering.

or silica NPs [8]. On the other hand, label-free detection techniques, including surface plasmon resonance (SPR), carbon nanotubes, microelectromechanical cantilevers and SELDI-TOF-mass spectrometry (MS) measure an inherent property of the query molecule, such as mass, dielectric or optical properties [9]. Several of these novel detection platforms have facilitated sensitive, specific, HT and rapid analysis of protein microarrays, thereby finding several applications in proteomics.

For any proteomics study to be successful, these emerging protein microarray platforms must be effectively coupled with sensitive and robust detection systems. This article provides an overview of the various protein microarray technologies and a comprehensive analysis on the progress of various protein microarray detection techniques, their merits, challenges and applications.

An overview of protein microarrays

Protein microarrays provide a valuable platform for both classical and functional proteome analysis and offer many advantages, the most important being HT analysis of thousands of proteins simultaneously. The construction of protein arrays is a significantly greater challenge compared with DNA microarrays due to: the lack of a PCR-equivalent amplification process, which can generate large amounts of protein; the wide variety of binding chemistries and specificities; the need to maintain structural integrity and association between subunits for functionality; differences in the physicochemical properties and size of proteins; and the complexity and variability introduced due to post-translational modifications of proteins in different expression systems. The central role played by proteins has nevertheless motivated researchers

to continuously develop and improve upon approaches to generate protein microarrays [10] for several applications, such as protein–protein interaction studies, biomarker detection studies and immunological studies, among others (TABLE 1) [4]. Based on their applications, the protein microarrays have been classified as analytical microarrays, reverse-phase microarrays and functional microarrays [11].

In analytical arrays, the affinity or capture agent such as an antigen or antibody is immobilized onto the solid array surface and incubated with the test sample. Targeted biomolecules are then captured on the array surface from the test lysate and can be detected by means of specific labeled secondary antibodies (FIGURE 2A). These analytical arrays are largely used for studying protein expression levels in complex mixtures and for measuring parameters such as binding affinity and specificity. Antibodies, despite being the most commonly used capture agents, tend to have some inherent drawbacks such as cross-reactivity and loss of activity upon immobilization, as is the

case for many other proteins [2]. Aptamers, which are short single-stranded oligonucleotides, are extremely promising molecules for the microarray format due to their stability, as well as their ability to bind to a wide range of target molecules with increased specificity and affinity [12]. Another alternative class of affinity reagents under investigation is the Affibody molecules, which consist of small protein bundles generated by combinatorial protein engineering with specificity for a wide range of targets including human, bacterial and viral proteins [13]. Capture arrays have found wide applicability in the detection of antigen–antibody interactions, biomarker detection [14,15] and immunological studies [16] and, more recently, to profile carbohydrate post-translational modifications (PTMs) on proteins using lectin microarrays, which could be extremely effective to study variations in specific glycans in the diseased state [17].

Reverse-phase protein (RPP) arrays are generated by immobilizing the test sample such as cellular lysate, tissue lysate or serum sample lysate on the array surface, which is then probed using a detection antibody against the target protein of interest. These antibodies are in turn detected by means of secondary tagging and signal amplification, if required (FIGURE 2B). The signal intensity of reverse-phase microarrays, also referred to as RPP blots, depends on the specificity of interaction between the analyte and the analyte-specific reagent, binding affinities, sterical accessibility and the concentration of analyte in the test sample [2]. The high effective concentration of analytes within a spot increases the tendency for spurious interactions and makes the detection of rare and less abundant proteins from a complex protein lysate more challenging due to interference from the copious proteins.

Table 1. Protein microarrays and their applications.

Technique/principle	Merits	Demerits	Applications	Ref.
Analytical microarray	Differential labeling for comparison Simple equipment	Chemical modification of sample Cross-reactivity Increased background signal	Protein interaction studies Biomarker detection Immunological studies	[4] [4,14,15] [4,16]
Reverse-phase microarray	Quick screening tool Simple and inexpensive Multiple sample analysis	Spurious interactions Narrow dynamic range Low-abundance protein detection not possible	Autoantibody profiling for prostate cancer PTM analysis Cancer signaling pathways	[19] [18] [20–22]
Functional microarray	HT protein production Stable protein microarrays	Tag interference with immobilization Can disrupt native conformation of protein	Study of PTMs Protein kinase substrate detection	[43] [42]
Microarrays using cell-based technology	Purified proteins HT analysis	Poor protein expression Protein insolubility Improper folding and PTM Laborious, time-consuming Poor shelf-life	Protein interactions Enzymatic reactions	[128] [129]
Cell-free expression-based microarray	Cloning not required (except NAPPA) Protein purification avoided On-demand synthesis; no storage concerns High-density arrays PTMs can be monitored	Proteins colocalized with DNA (in NAPPA) Possible loss of function due to tag sequence	Biomarker detection in cancer and autoimmune diseases Immunological studies Vaccine development Protein–protein interactions Toxin detection	[28,31] [32] [33] [34] [35]
Suspension array technology	Easy preparation and use High-density array Multiplexed analysis Less sample volume Flexibility	Laser may interfere with interaction High cost Cumbersome immobilization procedures	Detection of antibodies in serum Cytokine detection Detection of soluble cytokines	[28] [130] [131]

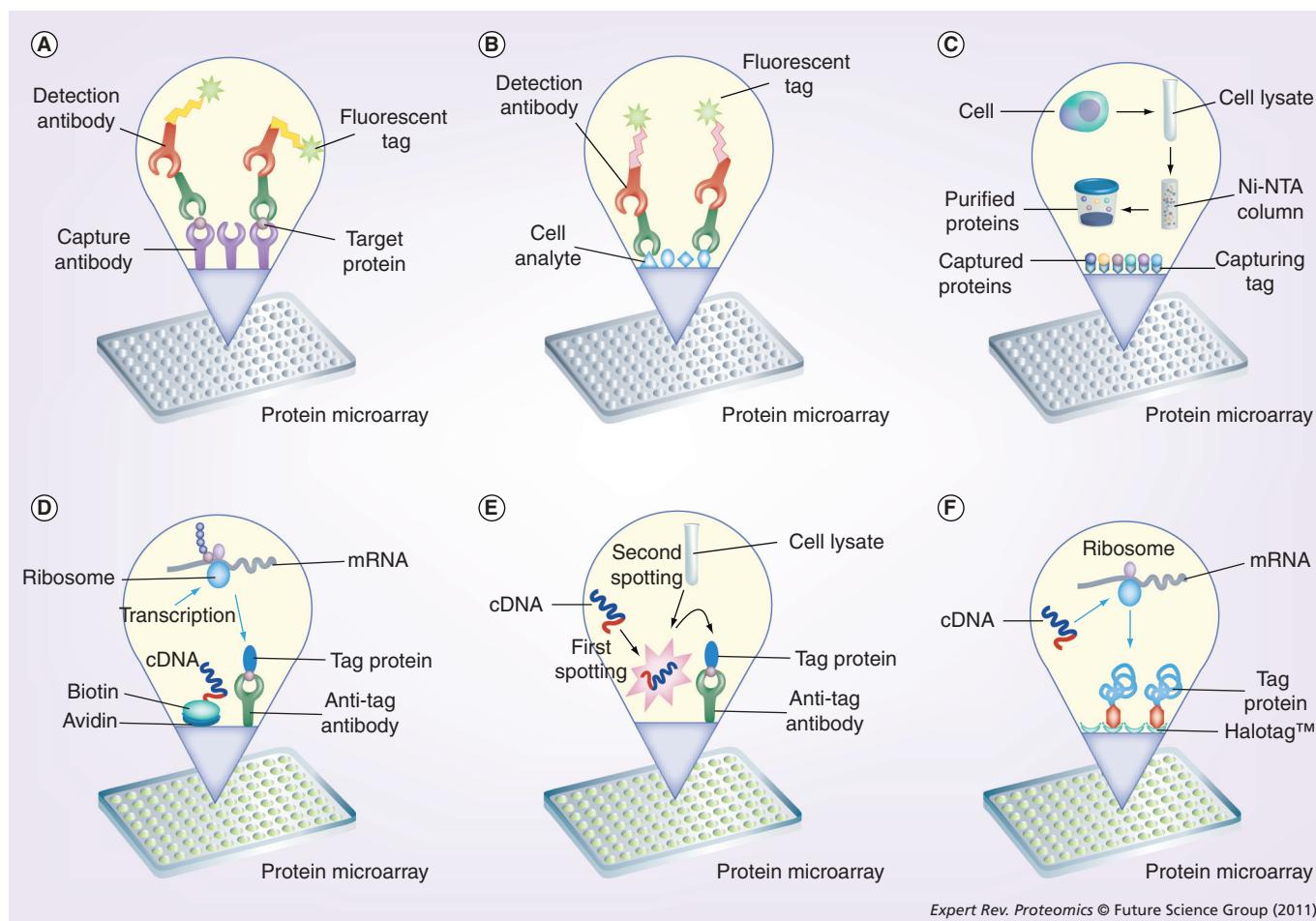
HT: High throughput; NAPPA: Nucleic acid programmable protein array; PTM: Post-translational modification.

This can be overcome to a large extent by prefractionation of the cell lysate before printing on the array. The advantage of RPP blots over analytical microarrays is that the proteins do not require labeling, but the disadvantage of RPP blots is that fewer analytes can be measured on a single array due to the limited availability of differentially labeled antibodies for detection. RPP arrays have been successfully used for analyses of PTMs and study of signal transduction in living cells [2,18], for profiling of autoantibodies in prostate cancer and benign prostatic hyperplasia [19], and for studying cell signalling pathways in ovarian [20], prostate [21] and breast cancer [22]. The ability of RPP arrays to generate a functional map of cell signalling pathways from biopsy specimens of patients has driven researchers to examine the potential of these arrays for personalized, patient-tailored therapies [23].

Functional microarrays study the biochemical properties and functions of thousands of native proteins, peptides or domains printed onto a chip surface in their active state after extensive purification using cell-based methods or by cell-free expression on the array [2]. Function-based arrays are generated by specific immobilization protocols involving various binding chemistries such that there is no loss of integrity or activity of the protein of interest [24]. An important factor for the success of functional protein microarrays is the availability of purified proteins for

immobilization (FIGURE 2C). Cell-based technologies involve the process of expression of proteins in heterologous host systems such as *Escherichia coli* and *Saccharomyces cerevisiae*, followed by their purification using suitable chromatographic and electrophoretic separation procedures [25]. The purified proteins are then printed onto the array surface to generate functional protein microarrays. Systematic cloning and expression of several open reading frames to generate a proteome chip can be a challenging task due to difficulties in expression of eukaryotic proteins, insolubility, improper folding and modifications in the host system, as well as other technical challenges such as maintenance of protein shelf life, stability and purity. Furthermore, the process is laborious and time-consuming, with multiple steps involved before the pure protein can be obtained [23]. Several protein expression systems have strived to continually improve and enhance key features of microarrays, such as the protein yield, proper folding, PTMs, speed, ease of use and reduced cost.

The drawbacks associated with protein microarrays generated by cell-based methodologies have been suitably overcome during the last decade by the development of cell-free expression microarrays (TABLE 1), which rely on *in situ* protein synthesis from their corresponding DNA templates using cell-free systems. Commonly used cell-free expression systems include *E. coli* S30, rabbit reticulocyte



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Figure 2. Different protein microarray formats employed for proteomic studies. (A) Capture arrays, wherein antibodies are immobilized on the array surface and incubated with test sample; detection is carried out by means of a labeled secondary antibody. (B) Reverse-phase arrays: test sample is immobilized on the array surface and probed using a suitable detection antibody. (C) Cell-based protein microarrays: the protein of interest is expressed in a heterologous host system, purified using Ni-NTA affinity column and then printed on the array surface for further studies. (D) Cell-free nucleic acid programmable protein array: the cDNA of interest is immobilized on the array slide and protein expression carried out by addition of cell-free lysate. The expressed protein is colocalized on the array through a tag-capturing agent. (E) Multiple spotting technique: the first step involves the spotting of template DNA followed by cell-free extract in the second spotting step. Expressed protein is captured on the array surface. (F) HaloTag™: proteins fused with HaloTag are expressed and covalently immobilized through the HaloTag ligand. Covalent capture ensures firm protein immobilization.

Ni-NTA: Nickel-nitrilotriacetic acid.

lysate and wheat germ extract. Protein *in situ* array, also known as DiscernArray™ technology, was the first technique of its kind described in 2001 [26], wherein protein arrays were generated in a rapid, single-step process by making use of PCR DNA constructs encoding the protein of interest along with required transcription and translation initiation sequences and a suitable N- or C-terminal tag sequence for immobilization. The coupled transcription and translation reactions were carried out in nickel-nitrilotriacetic acid-coated microtiter plates, onto which the expressed proteins were bound as soon as they were synthesized. Although these were solution-based reactions, the concept of *in situ* protein synthesis and subsequent immobilization to generate an array on planar surfaces originated here. Recently, many suspension array technologies, such as bead-based array technology [27] and Luminex xMAP® multiplexed suspension array technology [28], have emerged

for proteomic applications. These suspension array technologies have been dominating the flat surface microarrays because of their ease of preparation and use, highly comparable analysis, high array density and flexibility [29]. The commercially available microsphere arrays have replaced ELISA for multiplexed analysis of immunoassays [30]. Many improved techniques such as nucleic acid programmable protein array (FIGURE 2D), multiple spotting technique (FIGURE 2E), DNA array to protein array and HaloTag™ (FIGURE 2F) have been developed after the success of protein *in situ* array technology for the generation of cell-free expression microarrays, each having its own advantages and limitations [4]. The nucleic acid programmable protein array approach has successfully been used for generation of high-density protein microarrays with up to 3300 spots/slide [31]. Cell-free-based protein microarrays have successfully found several novel applications, such as early detection

of biomarkers [28], immunological studies [32], vaccine development [33] and identification of protein–protein interactions [34], as well as toxin detection [35].

Functional protein microarrays have also found many applications, one of the most important being the detection of post-translational modifications of proteins [36,37]. In 2005, Ptacek *et al.* developed a protein microarray immobilized with approximately 4400 proteins that were potential substrates for protein phosphorylation [37]. These substrates were then incubated with kinase and ATP, after which they were washed thoroughly to remove any unbound material. Substrates recognized by 87 different yeast kinases were identified, with over 4000 phosphorylation events involving 1325 different proteins being studied. These studies help in providing an insight into important biological aspects of protein networks and interactions [37].

Detection techniques for protein microarrays

In order to keep pace with rapid advancements in protein microarray technology, detection systems have also been significantly improved. Important parameters critical for the success of various sensing technologies are their sensitivity, limit of detection, dynamic range, multiplexing capability and resolution. The two major classes of detection techniques commonly used for microarray analysis are label-based (TABLE 2) and label-free systems (TABLE 3). While the label-based systems involve the use of a tag in the form of fluorescent dyes and radioisotopes, among others, for the query molecule, the label-free detection techniques measure an inherent property of the query such as mass, dielectric or optical properties. There have been numerous advancements in both label-based and label-free technologies, which are reviewed in detail in this section.

Label-based detection

A majority of the microarray applications to date have employed label-based detection techniques due to their ease of use, common availability of reagents and simple instrument requirements. In addition to the conventional labeling strategies such as use of radioisotopes [6], fluorescent dyes [5], chemiluminescent molecules and others, many novel labels such as inorganic QDs, gold NPs [7], dye-doped silica NPs [8], single-walled carbon nanotubes [38] and ultrasensitive bio-barcodes [39,40] have been developed for label-based detection in the microarray format. Certain bead-based techniques such as flow cytometry [41] and magnetic bead detection [42,43] have also been explored recently and have demonstrated promising results.

Conventional fluorescence labeling

The earliest labeling procedure for detection purpose was the use of radioisotope molecules like ^{32}P , which have been successfully employed to study protein–protein, protein–DNA, protein–RNA and protein–ligand interactions; however, radioisotope labeling has gradually declined over the years due to health and safety concerns and problems of waste disposal [6]. A convenient alternative that emerged and has been widely adopted is fluorescence labeling. Dyes such as fluorescein, rhodamine (Texas Red), nitrobenzoxadiazole, phycobiliproteins, acridines, BODIPY and cyanine compounds are

most commonly used for protein microarray detection. Factors governing the choice of fluorophore molecule to be used for detection include the sample type, substrate, light emission spectra characteristics and the number of target proteins to be studied [5]. Fluorescence detection is not compatible for detection of all substrates due to the inherent autofluorescence property of some materials, which decreases the signal-to-noise ratio. Cyanine dyes, Cy3 and Cy5, are among the most commonly used fluorophores for protein microarray detection because of their decreased dye interaction, brightness and ability to easily label proteins with charged lysine residues.

Detection by fluorescence labeling can be carried out either by direct labeling (one antibody assay) or indirect labeling [2]. In the direct labeling method, the target protein is labeled directly with a fluorophore (Cy3 or Cy5), which is captured by immobilized antibodies on the array surface. This technique permits coincubation of a reference sample with an analyte of interest, both having different tags, thereby facilitating internal normalization [44]. Direct labeling requires only a single capture antibody, has the capacity for multiplexed detection of hundreds of analytes, and offers accurate and reproducible results for abundant proteins. The drawbacks of direct labeling include low sensitivity, lack of specificity, cross-reactivity, chemical modification of the sample, and disruption of interactions between the target and captured antibodies due to the label [45]. A dual fluorescence method has been used for the detection of biomarkers in prostate cancer; serum samples from 33 patients and 20 healthy controls analyzed on a microarray immobilized with 180 antibodies revealed that nine proteins were found only in prostate cancer patients [44]. Srivastava *et al.* made use of Cy3 and Cy5 fluorophores for the identification of serum proteome signatures in cystic fibrosis (CF). Comparison of the protein expression patterns of pooled serum from CF patients and controls showed that CF patients were found to have selective expression patterns in the lungs and intestine [46]. Detection limits down to 6.25 pg have successfully been achieved using the Cy dyes [47].

In the indirect labeling method, unlabeled target proteins are captured by antibodies immobilized on the array surface. Detection is then carried out by means of a secondary antibody attached to a fluorophore molecule. This technique offers higher specificity due to binding of two target antibodies at different epitopes to the analyte of interest and high sensitivity because of low background labeling [45]. The specificity and sensitivity can further be enhanced by using a third antibody; however, this can also lead to increased detection of nonspecific signals due to the conserved regions shared by captured, as well as secondary, antibodies. The use of sandwich assay for multiplexed detection is usually limited to 30–50 targets due to a lack of specific antibodies for all purified antigen targets, which leads to cross-reactivity [45]. Biotin labeling, together with horseradish peroxidase-conjugated streptavidin, has been used for effective detection of vitamin E-regulated cytokines by means of a cytokine microarray [48]. The authors found that monocyte chemoattractant protein 1 was the most important target for vitamin E. ErbB2, a receptor tyrosine kinase, acts as a potent oncogene whose phosphorylation regulates cellular proliferation. Antibody

Table 2. Label-based detection techniques and applications.

Technique	Sensitivity	Merits	Demerits	Applications	Ref.
<i>Conventional fluorescence methods</i>					
Direct labeling	6.25 pg [47]	Only single Ab required High reproducibility Highly sensitive for abundant proteins Multiple sample assay	Low sensitivity to low-abundance proteins Cross-reactivity Chemically modified sample	Biomarker detection in prostate cancer and cystic fibrosis	[46]
Indirect labeling	NA	Greater specificity High sensitivity	Cross-reactivity Multiplexed analysis not possible High cost	Cytokine detection Small molecule inhibitors for signal transduction	[48] [49]
RCA	fM [132]	High sensitivity Reproducibility Broad dynamic range Multicolor detection Detection of low-abundance proteins	Critical validation procedures Higher variations due to different incubation times Decrease in robustness	Study of cytokine secretion	[50]
<i>SERS-based methods</i>					
Raman dye-labeled NPs	10–100 fg [54]	High sensitivity Flexibility due to nonoverlapping probes Sharp scattering peaks Cost effective	Complexity in synthesis of NPs Lack of uniformity	Protein–protein interaction and kinase substrate studies	[53,54]
SWNTs	1 fmol [38]	High sensitivity Multiplexed detection Minimum background signal Resistance to photobleaching	Metal impurities interfere with activity Insoluble in biological buffers Difficult to determine degree of purity	Autoantibody detection	[38]
Gold NPs	3–300 amol [56]	Improved optical property Superior quantum efficiency Compatible with wide range of wavelengths Resistance to photo bleaching	High cost Cytotoxicity Non-uniform size and shape of NPs	Multiplexed detection of PSA Carbohydrate–lectin and glycoprotein–lectin interactions Screening of antibacterial drugs	[56] [58] [59]
Quantum dots	pmol–fmol [64]	Brighter fluorescence Excellent photostability Multicolor fluorescent excitation Greater quantum yield	Toxicity Unknown mechanism	Cancer biomarker detection Detection of cancer-specific cytokines	[62,63] [64]
Dye-doped silica NPs	0.1 ng/ml [68]	Biocompatible High sensitivity Minimal aggregation and dye leakage Photostability High capacity	Complex synthesis process	Biomarker detection Detection of AFP	[68] [71]
Bio-barcode NPs	5–300 amol [40]	High sensitivity Less detection time Easy adaptability to multiple targets	Can only be used with known Abs	Biomarker detection Detection of PSA in prostate cancer	[74] [40]

Ab: Antibody; AFP: α -fetoprotein; HT: High throughput; NA: Not available; NP: Nanoparticle; PSA: Prostate-specific antigen; RCA: Rolling circle amplification; SERS: Surface-enhanced Raman scattering; SWNT: Single-walled carbon nanotube.

Table 2. Label-based detection techniques and applications (cont.).

Technique	Sensitivity	Merits	Demerits	Applications	Ref.
<i>Detection methods for bead-based suspension array technology</i>					
Flow cytometry	pM [78]	Speed and accuracy Reproducibility Less sample required Cost effective Highly sensitive Multiparameter detection	Multiplexed analysis not possible due to lack of matched-pair Abs and matrix High background noise	Measurement of various analytes HT biological testing	[76,77] [76]
Magnetic bead-based detection	NA	Quick assay Wide dynamic range Reproducibility Cost effective High specificity	Low sensitivity	Detection of autoantibodies Viral pathogen detection	[42] [79]

Ab: Antibody; AFP: α -fetoprotein; HT: High throughput; NA: Not available; NP: Nanoparticle; PSA: Prostate-specific antigen; RCA: Rolling circle amplification; SERS: Surface-enhanced Raman scattering; SWNT: Single-walled carbon nanotube.

microarrays were successfully used to monitor the activation, uptake and signal transduction of ErbB receptor kinases and also for the identification of small-molecule inhibitors of signal transduction pathways [49].

Abundance of the analyte of interest is often a concern during microarray detection studies due to the relatively low sensitivity of fluorescent dye molecules. The rolling circle amplification (RCA) method is a promising approach for on-chip signal amplification to improve the limits of detection. In RCA, the capture antibody spotted on the array binds to the analyte of interest, after which detection is carried out using a biotin-labeled secondary antibody. This is further detected by means of an anti-biotin antibody, which is conjugated with single-stranded DNA that is pre-annealed with circular DNA (FIGURE 3A) [50,51]. Zhou *et al.* developed a two-color RCA protocol for the detection of various labeled proteins from two serum samples that were captured on antibody microarrays. This two-color RCA method produced 30-fold higher fluorescence than direct and indirect labeling approaches [52]. The advantages of RCA compared with direct and indirect fluorescent labeling are its high sensitivity, broad dynamic range, reproducibility and multicolor detection [50,52]. However, signal amplification also tends to decrease the robustness and increases variation in detection due to which it is not always preferred. Secretion of several inflammatory cytokines, including macrophage inflammatory protein-1 β , IL-8, interferon-inducible protein-10, eotaxin-2, I-309 and macrophage-derived chemokine have, nevertheless, been successfully studied by means of a protein microarray printed with 75 cytokines using the RCA method [50] in which the authors successfully achieved detection limits down to the femtomolar level. With the RCA technique finding increasing number of applications, fluorescence labeling studies promise to offer improved detection sensitivities for proteomics applications.

Surface-enhanced Raman scattering-based methods

Light that is incident on an atom or molecule is typically scattered back with the same energy and, therefore, wavelength is the same. However, the Raman effect prevails in a small fraction

of the photons, whereby the energy of the scattered photon is different from that of the incident photon. Improved optical properties are therefore obtained due to the enhanced electromagnetic field at the surface of the particle, which is revealed using spectroscopic techniques like surface-enhanced Raman scattering (SERS). Surface-enhancing agents like gold or silver as well as functionalization with target molecules, augment the sensitivity of Raman spectroscopy, which is otherwise a weak process with the ability to scatter only one in 10^6 photons.

The Raman dye labeling method involves coating of the analyte or antibody along with the Raman dye directly onto a gold NP probe surface, which is used for detection purposes [53,54]. Visualization is carried out by staining with silver enhancement solution and hydroquinone. This technique is very sensitive compared with the conventional fluorescence methods and the spots can be further studied by Raman spectrometry coupled with fiber optic microscopy [55]. In 2008, Li *et al.* demonstrated the use of SERS assay by detecting well-known biomolecular interactions of IgG–protein A and biotin–avidin down to concentrations of 10 and 100 fg per microarray spot, respectively [54]. Another such SERS-based assay was employed to study kinase functionality and inhibition by making use of biotinylated anti-phosphoserine antibodies, which were probed with avidin-conjugated fluorescein. In the proof-of-concept study, the H89 inhibitor exhibited highly selective inhibition towards protein kinase A [54]. Compared with fluorescence-based detection methods, the Raman dye-labeled gold nanoprobes offer advantages such as high sensitivity, cost-effectiveness, greater flexibility due to the large pool of nonoverlapping probes and high selectivity, as well as fluorescence quenching [54].

Single-walled carbon nanotubes (SWNTs) have been found to exhibit distinct electrical and spectroscopic properties with a strong, characteristic resonance Raman signature. SWNTs provide high sensitivity, multiplexed detection of proteins, minimum background signal due to sharp scattering peaks and high signal-to-noise ratio that is essential for improved sensitivity of

Table 3. Label-free detection technique and applications.

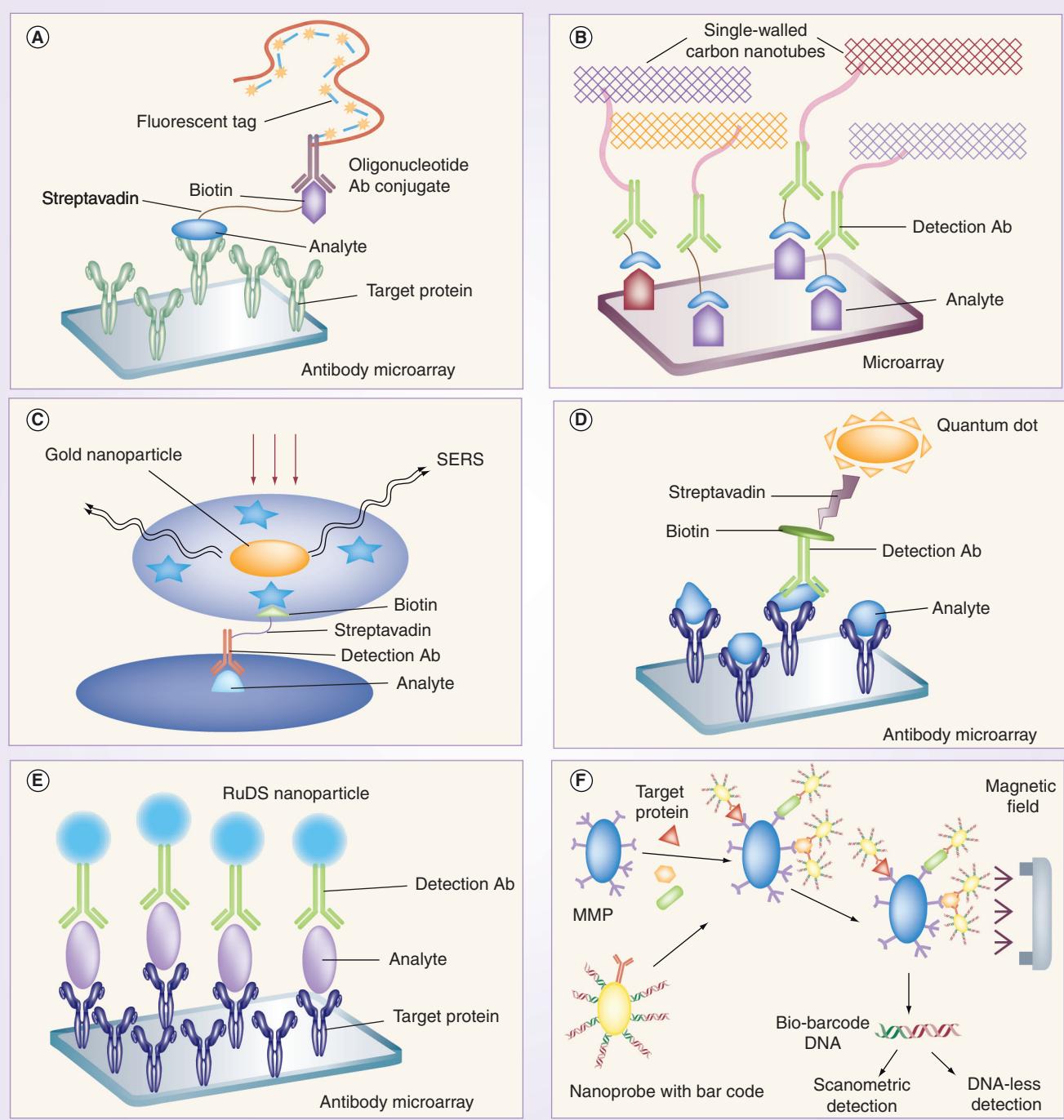
Technique	Sensitivity	Merits	Demerits	Applications	Ref.
Surface plasmon resonance	10 ng/ml [133]	Real-time detection High sensitivity Direct and rapid detection Multiplex studies	Limited to gold/silver array surfaces	Antibody detection and cancer diagnosis Detection of bacteria Biomarker detection in serum On-chip enzymatic reactions	[85] [84] [88] [89]
Nanotubes and nanowires	0.25 ng/ml [92]	Multiplexed analysis High specificity Robust nature	Limited to systemic studies Lack of established surface modifications Lack of control on synthesis	Detection of cancer biomarker Protein–small-molecule interactions Detection of single virus particle Pathogen detection Biological imaging Detection of biomolecules, immunoglobulins and pathogenic bacteria	[92] [98] [99] [93] [94] [95,96]
Micro cantilevers	40 nmol [134]	Short assay time Real-time detection Cost effective	False results with serum and complex samples	Ag–Ab binding assays Protein conformational change studies Study of complex biological samples Membrane protein–ligand interaction studies Biomolecular drug–target interaction Diagnostic studies	[105] [106] [107] [102] [103] [104]
Atomic force microscopy	0.025 pg/ml [135]	Original physiological conditions retained High specificity	Cannot be used for aqueous solution Artifacts	Protein–protein interactions	[117,118]
SELDI-TOF	1 fmol [136]	High specificity Effective for high-abundance proteins	Inability to detect tightly bound proteins Accessibility to low-molecular-weight proteins Inability to identify proteins after recovery	Diagnosis of various cancers Protein profiling from dry eye Biomarker detection Screening of proteins in hepatocellular carcinoma	[113] [111] [109,110] [112]
Scanning tunneling microscopy	100 fg/ml [120]	Direct response Cost effective Integration with semiconductor technology Highly sensitive Detection extendable to nanoscale array	Multiplexed detection not possible	Detection of HSA Detection of PSA	[120] [121]
Spectral domain optical coherence phase microscopy	5 pg/mm ² in average five spots [127]	High spatial resolution High-density protein microarrays	Not suitable for diagnostic sensor application	Detection of multiplexed biomolecular interactions	[127]

Ab: Antibody; Ag: Antigen; HSA: Human serum albumin; PSA: Prostate-specific antigen.

detection. Macromolecular SWNTs functionalized with specific Raman-labeled antibodies have been used for multiplexed detection of target proteins bound on a gold-coated microarray slide (FIGURE 3B). Chen *et al.* made use of this technique for detection of autoantibodies against proteinase 3, a biomarker for Wegener's granulomatosis. Detection limit down to 1 fM, which is 1000-fold more sensitive than conventional fluorescence, was successfully achieved using this protocol [38]. The high Raman scattering cross-section, increased stability and simple and tunable spectra have made SWNTs an attractive option for SERS-based protein detection studies.

Gold NPs

The excitation of coherent electron oscillations that exist at the interface between two materials is known as SPR and forms the basis for use of gold NPs as label-based detection systems. The proportion of light absorption to scattering is dependent on the size of the NP, with larger NPs being used for biological imaging due to the requirement for a high scattering cross-section. Gold NPs, labeled with the suitable capture biomolecule, exhibit a change in emission spectrum of scattered light upon binding to the analyte of interest from a protein mixture by means of specific biomolecular interactions (FIGURE 3C) [7]. Gold nanoshells consist of spherical NPs



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Figure 3. Label-based detection strategies for protein microarray applications. **(A)** Rolling circle amplification: capture antibody spotted on the array surface binds to the analyte of interest and is detected by biotin-labeled secondary antibody. This is then detected by oligonucleotide-linked antibiotin antibody. **(B)** SERS-based single-walled nanotubes: macromolecular single-walled carbon nanotubes functionalized with Raman-labeled antibodies used for multiplexed detection of target proteins bound on a gold-coated array surface. **(C)** Gold nanoparticles (NPs): change in emission spectrum of scattered light detected upon binding of the gold NP-conjugated antibody to the analyte of interest. **(D)** Quantum dots: change in optical properties due to formation of excitons upon binding of quantum dot-conjugated antibody to target analyte. **(E)** Dye-doped silica NPs: silica-based NPs with large amounts of fluorescent dye packed inside them can be used to selectively label probe molecules for detection of interactions. **(F)** NP based bio-barcode: NP probes encoded with DNA unique to the protein of interest and suitable antibodies capture the MMPs with antibodies for the target analyte, thereby sandwiching the target protein. These are magnetically separated, the oligonucleotides dehybridized and are then sequenced to identify the protein of interest. Ab: Antibody; MMP: Magnetic microparticle probe; RuDS: Rubpy-doped silica; SERS: Surface-enhanced Raman scattering.

with a dielectric core NP, typically gold sulfide or silica, coated with a thin gold shell. The absolute size of these gold nanoshells can be manipulated such that they either strongly absorb or scatter the incident light; this in turn can alter the plasmon resonance such that the wavelength is shifted from visible to mid-infrared region.

Gold NPs have found wide applications for sensitive detection of standard proteins. They provide improved optical property, superior quantum efficiency, compatibility with a wide range of wavelengths and chemical stability against photobleaching [7]. Scanometric immunoassay with gold NP probes were used for multiplexed detection of prostate-specific antigen (PSA) with improved detection sensitivity on protein microarray compared with silver enhancement, with a detection limit of 300 amol in buffer and 3 fmol in 10% serum [56]. Colorimetric gold NP probes conjugated with antibodies and coupled with silver enhancement have been used for the detection of analytes on microarrays with sensitivity down to 1 pg [57]. Carbohydrate–protein interactions, which are more difficult to monitor than protein–protein interactions, have been studied using a microarray-based resonance light-scattering approach. Sensitive and selective assays were developed for the detection of carbohydrates, lectins and glycoproteins using gold NPs followed by silver deposition for signal enhancement, and proof-of-concept experiments successfully demonstrated the carbohydrate–lectin and glycoprotein–lectin interactions [58]. This technique has also been innovatively applied for the extremely selective discrimination between various bacterial strains with sensitivity down to 10^3 cells/assay and a dynamic range covering nearly three orders of magnitude, as well as for screening of antibacterial drugs [59]. Although gold NPs have been reported to be suitable for *in vivo* studies due to their biocompatibility and low cellular toxicity, systematic cytotoxicity studies must be performed every time the NP is functionalized with a different ligand.

Quantum dots

Quantum dots are nanometer-sized crystals composed of a semiconductor fluorescent core (cadmium selenide) coated with another semiconductor shell (cadmium sulphide or zinc sulphide) with a larger spectral band gap, and stable light-scattering or -emitting properties. Formation of excitons takes place when light of higher energy than that of the bandgap of the composing semiconductor is incident on the QDs. When these excitons return to their lower energy level, emission of a narrow, symmetric energy band takes place (FIGURE 3D) [7,60]. QDs find a diverse range of applications in biological sciences such as diagnostic imaging [61], detection of cancer biomarkers [62,63], direct probing of human serum [64] and tumor biopsy analysis [65]. The key advantages of QDs compared with organic dyes include bright fluorescence, excellent photostability against photobleaching, multicolor fluorescence excitation, tunable emission spectra even with single excitation wavelength, narrow emission spectra and greater quantum yield [7].

A sensitive and selective microfluidic protein chip was developed by Hu *et al.* in 2010 for multiplexed assay of cancer biomarkers [63]. The authors made use of IgG-conjugated QDs, which could be used for detection purposes in both sandwich and reverse-phase assays. Aqueous phase-synthesized cadmium

telluride/cadmium sulphide QDs were used as fluorescent signal amplifiers, which helped in improving sensitivity to femtomolar levels and allowed the probe to be used directly with serum. In 2007, Zajac *et al.* innovatively made use of streptavidin-coated QDs for detection of six cancer-specific cytokines using antibody microarrays. TNF- α , IL-8, IL-6, macrophage inflammatory protein-1 β , IL-13 and IL-1 β were successfully detected down to picomolar concentrations [64]. Pegylated, streptavidin-conjugated QDs were also found to have superior detection characteristics over the nonpegylated form in assays performed on reverse-phase protein arrays using cellular protein extracts [66]. A comparative study between QDs and various organic fluorophores revealed QDs to be 30-times more sensitive [62]. The authors successfully used QD labeling for identification of three important cancer biomarkers, carcinoembryonic antigen, cancer antigen 125 and Her-2/Neu in serum, as well as in saliva. Despite the benefits offered, the low stability and short lifetime of QDs due to their susceptibility to undergo oxidation and photolysis pose tremendous hazards not only to human health but also to the environment. Toxicity of QDs, caused primarily due to the cadmium ions present in the QD core, is a serious concern that needs to be addressed before they can find widespread biomedical applications [63,67].

Dye-doped silica NPs

Silica-based nanomaterials, which have a large quantity of fluorescent dye packed inside the silica matrix, have the ability to selectively tag a wide variety of biologically important targets such as cancer cells, bacteria and other biomolecules (FIGURE 3E). Silica NPs are nontoxic, biocompatible, environment-friendly, easy to modify and recover, resist microbial growth and are not affected by changes in pH. High sensitivity, minimal aggregation and dye leakage, the ability to trap hundreds of dye molecules in a single silica NP, brightness and photostability are some of the other advantages offered by these dye-doped labeling molecules [8]. Application of various functionalized silica NPs have been demonstrated in diverse fields such as biomarker discovery [68], drug delivery [69] and multiplexed signaling in bioanalysis [69]. Rubpy-doped silica NPs that were covalently modified with mouse antihuman CD-10 antibodies were incubated with mononuclear lymphoid target cells and used for selective detection of target leukemia cells [70]. Recently, rubpy-doped silica dye-encapsulated silica NPs were also used as a label for detection of IL-6 biomarker on protein microarray [68]. These results indicated that sensitivity down to 0.1 ng/ml could be achieved with high specificity towards IL-6 in the presence of a range of other cytokines and proteins. A novel class of near-infrared fluorescent NPs have also been successfully employed for the development of an immunoagglutination assay for detection of α -fetoprotein in whole blood samples using fluorescence-anisotropy measurement [71], clearly suggesting that they hold great potential for HT clinical and diagnostic studies in the microarray format.

NP-based bio-barcodes

A highly effective bio-barcode technique for ultrasensitive protein detection was developed by Mirkin's group in 2003 with the help of suitably functionalized NPs [39]. This bio-barcode technology

made use of magnetic microparticle probes (MMPs) containing antibodies specific to the target of interest and NP probes encoded with DNA that is unique to the protein of interest along with antibodies capable of sandwiching the proteins captured by the MMPs [39,72]. The NP probe–target–MMP complex was magnetically separated, after which the oligonucleotide strands were dehybridized and sequenced to identify the target protein (FIGURE 3F). Significant amplification was feasible using this technique, with detection limits down to 30 attomolar (aM) being achieved, which could further be boosted to 3 aM by means of PCR. The liberated oligonucleotide barcodes can be identified on microarrays by scanometric detection, by conventional markers like fluorophores [73], chemiluminescent probes and Raman dyes.

Quick assaying due to homogeneity of target binding, increased sensitivity, improved selectivity and easy adaptability are some of the advantages offered by bio-barcodes. One of the drawbacks of this system, however, was that only known antibodies could be used for detection purposes [39,72]. Bio-barcodes have been successfully employed for multiplexed detection of cancer markers [74]. In 2009, Thaxton *et al.* carried out a clinical pilot study for detection of PSA in serum of men who had undergone radical prostatectomy for prostate cancer. The barcode assay, which was found to be approximately 300-times more sensitive than other commercial immunoassays, promises to be a vital tool for disease diagnosis, prediction of recurrence and also to follow the prognosis of a patient in response to therapy [40].

Detection methods for suspension array technology

Flow cytometry

Flow cytometry, which can be considered as a very specific form of fluorescent detection technique, offers an established platform for rapid, quantitative and multiplexed detection of analytes in biological samples. Microspheres (microbeads) of different colors and sizes containing bound capture antibodies can be incubated with target proteins from various samples, which are then reacted with fluorescent-tagged secondary antibodies [30]. Detection is carried out by passing the beads through a flow cytometer that contains two laser sources – one for detection of captured antibodies on the colored bead and a second for quantitative and qualitative detection of analyte bound to the bead through the tagged secondary antibody. Speed and accuracy of analysis, lower background signal due to only a small sample volume being excited by laser, reproducibility, rapid detection of multiple samples, cost-effectiveness and high sensitivity are some of the advantages of flow cytometry over conventional fluorescent detection [41].

A rapid detection technique was developed by Faucher *et al.* in 2003 for simultaneous analysis of up to seven recombinant HIV-1 proteins using a protein bead array. The recombinant proteins were covalently coupled to color-coded microfluorospheres, which were then incubated with plasma and dried blood spot specimens for 1 h. HIV-1-specific antibodies were detected using biotinylated anti-human IgGs and streptavidin–phycoerythrin conjugates and the results were found to be comparable to those obtained using commercial enzyme immunoassays [75]. The cytomeric bead array has been used to design assays for measurement

of several analytes, including inflammatory mediators, cytokines, chemokines, intracellular signaling molecules and apoptotic factors, which position it as an attractive platform for robust, HT biological testing and drug discovery [76,77]. In addition, several studies have shown that assays performed using the multiplexed bead array provide results that are comparable to those achieved using ELISA [29]. Antibody based suspension bead arrays, which make use of color-coded beads for creation of the antibody arrays, offer a powerful tool for rapid, systematic and multiplexed analysis of a large number of serum samples with sensitivity down to the picomolar level and a broad dynamic range [78]. Bead-based assays using flow cytometry promise to provide a versatile platform for numerous protein microarray applications.

Magnetic bead-based detection

Magnetic bead-based detection technology, in which secondary antibodies coated on magnetic beads are used to detect target proteins on the capture antibody, provides a convenient alternative to fluorescent labeling [42]. High sensitivity and reproducibility, rapid detection, low background signal due to removal of weakly bound beads, wide dynamic range, high specificity and cost-effectiveness make this an ideal detection method for various suspension array technologies [42,43]. Leblanc *et al.* developed an innovative platform for simple and simultaneous detection of four pestiviruses of the *Flaviviridae* family [79]. This technique consisted of an oligonucleotide array hybridized with 5' biotinylated PCR products, which was detected by means of streptavidin-coated magnetic particles and could be visualized either with the naked eye, microscope or biochip reader [79]. Genotyping study of single-nucleotide polymorphism variants [80] and protein microarray analysis along with on-chip electrophoresis [43] are some of the other successful applications of this technique. A comparison of magnetic bead-coated antibodies with the traditional fluorescence method for the detection of 12 autoantigens from 21 serum samples from patients with autoimmune disease and healthy controls on an antigen microarray revealed that magnetic bead detection is a highly sensitive and cost-effective method [42]. This technique is, however, still at a proof-of-concept level and requires much further work before it becomes established.

Label-free detection

The limitations posed by the label-based detection strategies such as the alteration of surface characteristics of the query molecule, time and effort required for the labeling procedure, difficulty in multiplexing and toxicity has increased interest in the use of label-free approaches, which rely on the measurement of an inherent property of the query molecule itself. The label-free techniques are capable of studying the kinetics of biomolecular interactions in real-time, and are not hindered by interference from tag molecules. Other prerequisites for these detection systems are compatibility with HT arraying methods, ability to detect binding of small molecules to protein targets and a wide dynamic range. However, they have not yet been widely adopted due to uncertainty regarding their sensitivity and specificity. A variety of technologies, such as SPR, atomic force microscopy (AFM), carbon nanotubes and

nanowires, micro-electromechanical cantilevers and SELDI are at various stages of development and are attempting to overcome the hurdles posed by label-based techniques [9,81].

Surface plasmon resonance

Surface plasmon resonance is based on the creation of surface plasmons, that is, oscillations of free electrons that propagate parallel to a metal/dielectric interface, and measures change in refractive index very close to the sensor surface [9]. Any adsorption or desorption of molecules at the surface is reflected in the sensogram by a change in the reflection intensity with respect to the incident angle (FIGURE 4A). SPR enables direct and rapid determination of association and dissociation rates of a binding process, determination of strength of the binding and specificity of interactions on a large scale. Moreover, it makes it possible to measure biomolecular interactions in real-time with a very high degree of sensitivity [81]. Coupling of a spatially resolved measuring device with SPR provides another innovative platform known as SPR imaging (SPRI) [82]. Silanized glass slides coated with gold NPs and the protein of interest have also been used for label-free detection studies by measuring the shift in wavelength of the localized SPR spectra, whose band responses can be measured in real-time using a UV-Vis spectrometer with a charge-coupled device array detector [83].

A label-free SPR immunosensor has been employed to detect bacteria *Listeria monocytogenes*, one of the most difficult pathogens to treat. Affinity purified monoclonal antibodies were used as capture antibodies for detection of *L. monocytogenes* cells bound on a gold substrate [84]. SPR is increasingly being used for several biomedical applications, such as antibody screening [85] and cancer diagnosis, as well as for innovative applications such as label-free detection of narcotics on a microarray chip [86]. In 2009, Ladd *et al.* made use of SPRI technique for the detection of potential biomarker candidates in cancer with the help of antibody microarrays [87]. SPRI is proving to be an effective technique for biomarker detection in serum proteomic studies using microarrays [88] and for the detection of on-chip enzymatic reactions using peptide arrays [89]. Microfluidics combined with SPRI detection has been developed to overcome the challenge of continuous introduction of different analyte concentrations during an experiment, which was tested using immobilized human- α -thrombin and with injection of anti-human α -thrombin IgG [90]. These studies lend support to the immense potential of SPR and its related techniques for sensitive protein microarray detection.

Nanotubes & nanowires

Carbon nanotubes are hollow, cylindrical graphite sheets that possess excellent mechanical strength and chemical stability and are classified as single-walled or multi-walled, depending on their graphite layer composition. Development of nanosensors specifically requires semiconducting carbon nanotubes, which is usually difficult to obtain due to lack of control in the synthesis process [7]. Their use as detection systems relies on the changes in electrical conductance observed when target molecules bind to the functionalized nanotubes or nanowires [81,91]. The integration of carbon

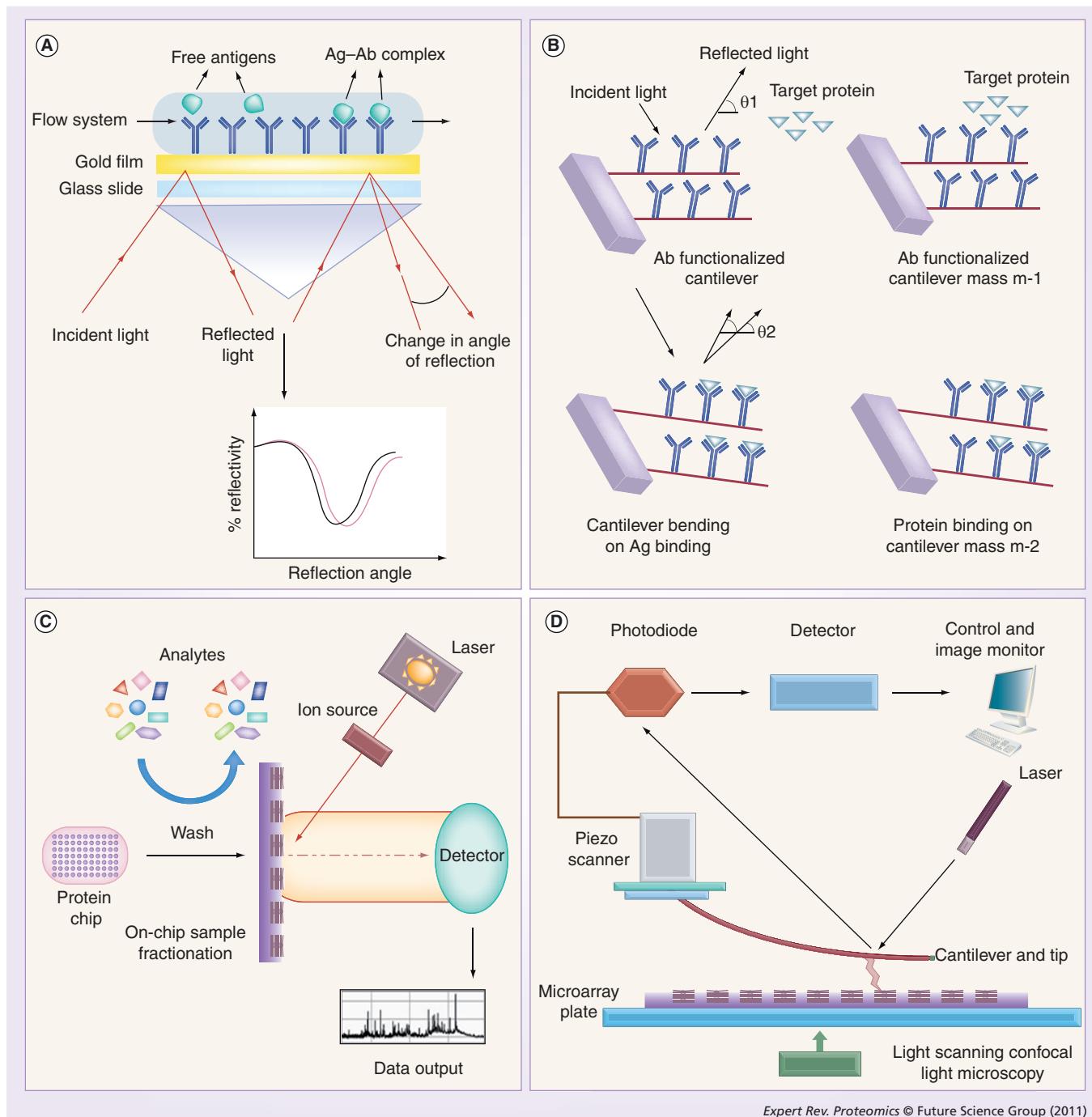
nanotubes with field-effect transistors has led to the development of carbon nanotube field-effect transistor biosensing devices, which have gained popularity due to their excellent sensitivity, cost-effectiveness and robust nature [7].

Several nanotube-based immunosensors have been developed for label-free detection of biological markers [92], pathogen detection and deactivation [93], and biological imaging studies [94]. Detection of total PSA (T-PSA) using differential pulse voltammetry was carried out by Okuno *et al.*, wherein the current signals obtained from oxidation of electrochemically active amino acids like tyrosine and tryptophan were used to determine the interaction between T-PSA and T-PSA antibody covalently immobilized on the SWNT array-modified microelectrodes. Sensitivity down to 0.25 ng/ml could be achieved with a signal-to-noise ratio of 3 [92]. An interesting application is seen in the study by Joshi *et al.*, wherein carbon nanotubes have been used for deactivation of anthrax toxin by making use of nanotube-mediated reactive oxygen species [93]. Carbon nanotube field-effect transistors have found applications for detection of biological molecules, immunoglobulins [95] and pathogenic bacteria [96]. However, application of carbon nanotube-based nanosensors for proteomic studies is still in its infancy and several issues regarding their toxicity and biocompatibility need to be overcome before they can be adopted for routine research.

Silicon nanowire field-effect transistors (SiNW-FETs), which are based on the same principle as carbon nanotubes, have also found many novel applications in proteomics. Zheng *et al.* developed a SiNW-FET biosensor by coupling monoclonal antibodies to the surface, which were employed for multiplexed detection of cancer markers in undiluted serum [97]. Detection limits achieved by this technique are believed to be approximately 10^3 times more sensitive than some of the other label-free detection techniques. Study of protein–small molecule interactions [98], real-time selective detection of single virus particles [99], and delivery of cytotoxic agents and antibiotics into target cells [100] are some of the other applications of SiNW-FETs.

Microcantilevers

Microcantilevers are thin, silicon-based, gold-coated surfaces that make use of nanomechanics for biomolecular recognition [101]. The silicon strips are attached at one end with the antibody or protein bound to its surface such that bending of the cantilever upon analyte binding can be measured by suitable optical or electronic systems (FIGURE 4B) [9,102]. The study of membrane protein receptor–ligand interactions, which is of immense medical relevance, is often a challenging task. In 2009, Braun *et al.* successfully made use of microcantilever sensors for label-free, time-resolved detection of membrane protein–ligand interactions in an array format [102]. Detection of various biomolecular and drug–target interactions [103], diagnostic studies [104], antigen–antibody binding assays [105], detection of protein conformational changes [106] and study of complex biological samples [107] are some of the successful applications of microcantilever sensors. Piezoelectric cantilever arrays with an electrical readout have also been applied for protein detection using glutathione-S-transferase (GST) and the detection of GST antibodies down to a concentration of 40 nM, which is comparable



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Figure 4. Label-free detection techniques using protein microarrays. **(A)** Surface plasmon resonance: any changes in refractive index at the surface due to binding interactions between the probe and analyte are reflected in the sensorgram by changes in reflection angle. **(B)** Microcantilevers: bending of the antibody-coated silicon cantilevers upon binding of the target analyte is detected either by a change in an optical property, such as a change in the angle of reflection, or by a change in an electronic property such as a change in mass. **(C)** SELDI-TOF-MS: target proteins captured on the chip are retained after washing and on-chip fractionation, after which they are subjected to detection using time-of-flight mass spectrometry. **(D)** Atomic force microscopy: schematic representation of instrumentation used for atomic force microscopy. Surface topological changes on the microarray plate can be detected with picometer resolution by measuring any deflection of the cantilever. Ab: Antibody; Ag: Antigen.

to the optical read-out system [108]. Short assay times and the use of uncomplicated fabrication methods make microcantilevers very promising tools for protein microarray-based applications.

SELDI-TOF MS

The concept of SELDI-TOF makes use of proteins bound on a solid-phase chromatographic surface with specific properties onto which complex biological mixtures (e.g., cell extracts) are added. Unbound proteins and other interfering compounds are washed off. The subset of proteins retained by the chip are subsequently ionized and detected by TOF-MS (FIGURE 4C). A unique fingerprint is generated for each sample and, consequently, patterns of masses are produced by SELDI analysis. This technique has demonstrated its advantages for quantitative profiling of tissue proteomes and on-chip HT sample processing. Zhang *et al.* made use of weak cation-exchange chips to analyze 140 serum samples from astrocytoma patients, controls and those with other brain tumors. Seven serum biomarkers were found to be significantly deregulated in the astrocytoma patients, which on further analysis by SELDI-TOF-MS revealed that four were upregulated and three were downregulated [109]. The SELDI protein chip technology has also found wide applications for detection of hepatitis B virus-induced liver cirrhosis through novel serum biomarkers [110], profiling of proteins in tears from patients with dry eye [111], screening for low-molecular-weight and low-abundance proteins in hepatocellular carcinoma [112], and in serum protein profiling studies for the diagnosis of various cancers [113]. Major drawbacks of the system, however, include the inability to detect proteins that are tightly bound to the plate surface, its suitability for profiling only low-molecular-weight proteins (<20 kDa) and the inability to identify proteins after subsequent recovery.

Atomic force microscopy

Atomic force microscopy is based on scanning probe microscopy and provides information on surface topological changes (FIGURE 4D). AFM finds applications in surface characterization of protein microarrays [114], immobilization of different biomolecules with micrometer resolution [115,116], as well as for the detection of protein–protein interactions [117,118]. A unique three-step technique for the immobilization of oligopeptide probes for protein microarray applications has been exhibited using a combination of AFM, fourier transform infrared spectroscopy and fluorescence scanning microscopy [116].

Other promising detection techniques

In addition to the various techniques discussed previously, there are several label-based and label-free detection methods that are in various stages of development and have shown promise for several applications. Dye-doped NPs coupled with antibodies have shown their application for ELISA, fluorophore-linked immunosorbent assay (FLISA), immunohistochemistry, immunocytochemistry and protein microarray studies [119]. Lanthanide-based NPs, which offer resistance to photobleaching, sharp emission profiles, long fluorescence lifetime and several other advantages, have shown encouraging results; however, their compatibility for microarray

studies still needs to be explored further [8]. Another convenient alternative to conventional fluorescence-based detection that has emerged recently is scanning tunneling microscopy, which carries out electrical detection of binding events on protein microarrays. A protein chip, which was fabricated using a microcontact technique on gold substrate and subsequently characterized by scanning tunneling microscopy, was shown to detect human serum albumin with sensitivity down to 100 fg/ml [120]. A similar study using this technique detected PSA at concentrations down to 33 fM [121], showing their great potential for protein microarray studies.

An emerging label-free approach for protein microarray studies is electrochemical impedance spectroscopy, which is performed in the presence of a redox probe and considered to be an effective method for sensing interactions on electrode surfaces [122]. Recent introduction of aptamers for electrochemical impedance spectroscopy-based biosensing studies has led to improved sensitivity of the technique [123] and promises to provide an important platform for microarray-based studies [124]. Some of the other promising label-free approaches include biosensor chip MS [125], spectral reflectance imaging biosensor [126,127] and spectral-domain optical coherence phase microscopy [127]. With a host of novel techniques being applied for protein microarray studies, researchers have the option of choosing the appropriate detection system based on their requirements.

Concluding remarks

Proteomics research is increasingly turning towards gel-free approaches such as protein microarrays as well as other techniques including tandem MS (LC-MS/MS) for quantitative as well as functional analysis. Protein microarrays allow for HT analysis of immobilized proteins, with whole-proteome assays being their ultimate goal. Abundance-based protein arrays, in the form of capture arrays or reverse-phase protein blots, have found extensive applications in immunological studies, diagnosis and monitoring of diseases like cancer, and interactions of proteins with peptides, small molecules and other biomolecules. Efforts to minimize nonspecific interactions and cross-reactivity have helped in improving the specificity of these arrays. Functional protein arrays have witnessed a slow transition from cell-based techniques to cell-free expression systems for the generation of microarrays. Cell-free technologies have adeptly overcome the hurdles posed by protracted cell-based methods such as expression in heterologous hosts, purification and need for storage, and have facilitated the parallel synthesis of several proteins on demand in a single reaction. Cell-free-based protein microarrays have been used for numerous applications, including biomarker discovery, vaccine development, immunological studies and protein interaction analysis.

Detection systems for protein microarrays have strived to achieve real-time, multiplexed detection of proteins and their interactions. Despite continuous advancements and increasing applications of label-based strategies, the focus has been shifting towards label-free approaches that achieve detection of target molecules by examining an inherent property of the query molecule itself, including mass, dielectric property, light scattering and refractive index. They have been able to overcome several of the limitations posed

by label-based approaches. Some of the emerging label-free techniques include SPRI-based systems, silicon nanowire field-effect transistors and carbon nanotubes and nanowires. These sensitive tools have been successfully applied to several proof-of-concept studies but their applicability to large, high-density microarrays is yet to be demonstrated. While protein microarrays and detection techniques have made rapid progress in past 5 years, further progress in detection techniques will provide enhanced sensitivity and specificity, and further miniaturized array formats.

Five-year view

Label-free detection systems have evolved in recent years and have been tested for various proteomic applications. However, despite these efforts, the label-based systems, particularly fluorescence-based detection, continue to be mainly used for proteomic studies due to ease of usage, cost-effectiveness and simpler instrument requirements. With increased sensitivity of these techniques, the research community has been reluctant to experiment with more complicated label-free technologies.

Nevertheless, going ahead, label-free systems will find widespread use for protein microarray applications due to their ability to carry out multiplexed detection. With an increasing number of proteins already being printed on array surfaces, whole proteome chips will soon be used for a large number of application studies. Use of label-free techniques for such high-density protein arrays will ensure proper protein folding and functionality due to the absence of an interfering tag. Improved sensitivity of label-free technologies will provide quantitative data such as affinity constants and kinetic parameters, among others, and further enhance

the quality of results that can be obtained. Most studies that have been performed until now have made use of ideal model systems for proof-of-concept experiments. This trend should gradually change in the years to come and label-free techniques will be used for the detection and study of more generic biological interactions and challenges. Increased adoption of these systems in different laboratories across the world will help in evaluating the true efficiency and variability of label-free detection.

Expert commentary

The increasing use of protein microarrays for proteomic studies has spurred the advancement of various detection technologies. With improved sensitivity limits now being achieved by label-based techniques, they continue to be the preferred method of detection due to rapid, simple and cost-effective assays. However, label-free systems are offering multiplexed detection without the interference of any tagging agent, thereby ensuring proper protein folding and functionality. Although label-free detection techniques have, until now, largely been demonstrated at the proof-of-concept level, they are increasingly gaining attention due to the benefits they offer.

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Key issues

- Cell-based technologies for printing protein microarrays are faced with poor protein expression, protein insolubility, improper folding and poor shelf life.
- Cell-free expression-based protein microarrays are not widely used but should be increasingly adopted in the future for the generation of high-density arrays.
- Low abundance of a protein of interest in complex samples such as serum poses a great challenge for detection systems.
- Spurious interactions and nonspecific detection of other analytes are problematic.
- Improving the sensitivity of detection and introducing more amplification systems will allow low-abundance proteins to be detected.
- The mechanisms of action are unknown in some of the newly developed label-based and label-free detection systems.
- Concerns exist regarding the toxicity of some systems and hazards posed to human health and the environment.

References

Papers of special note have been highlighted as:

- of interest
- of considerable interest

- 1 Hu Y, Huang X, Chen GY, Yao SQ. Recent advances in gel-based proteome profiling techniques. *Mol. Biotechnol.* 28(1), 63–76 (2004).
- 2 Labaer J, Ramachandran N. Protein microarrays as tools for functional proteomics. *Curr. Opin. Chem. Biol.* 9(1), 14–19 (2005).
- 3 Templin MF, Stoll D, Schwenk JM, Potz O, Kramer S, Joos TO. Protein microarrays:

promising tools for proteomic research. *Proteomics* 3(11), 2155–2166 (2003).

- 4 Chandra H, Srivastava S. Cell-free synthesis-based protein microarrays and their applications. *Proteomics* 10(4), 717–730 (2010).
- 5 Espina V, Woodhouse EC, Wulfkuhle J, Asmussen HD, Petricoin EF III, Liotta LA. Protein microarray detection strategies: focus on direct detection technologies. *J. Immunol. Methods* 290(1–2), 121–133 (2004).
- 6 Angenendt P. Progress in protein and antibody microarray technology. *Drug Discov. Today* 10(7), 503–511 (2005).
- 7 Ray S, Chandra H, Srivastava S. Nanotechniques in proteomics: current status, promises and challenges. *Biosens. Bioelectron.* 25(11), 2389–2401 (2010).
- 8 Yan J, Estévez MC, Smith JE *et al.* Dye-doped nanoparticles for bioanalysis. *Nano Today* 2(3), 44–50 (2007).
- 9 Ray S, Mehta G, Srivastava S. Label-free detection techniques for protein microarrays: prospects, merits and challenges. *Proteomics* 10(4), 731–748 (2010).
- 10 Wolf-Yadlin A, Sevecka M, MacBeath G. Dissecting protein function and signaling

- using protein microarrays. *Curr. Opin. Chem. Biol.* 13(4), 398–405 (2009).
- 11 Hall DA, Ptacek J, Snyder M. Protein microarray technology. *Mech. Ageing Dev.* 128(1), 161–167 (2007).
- Provides a concise overview of different protein microarrays and protein chips, along with the associated detection systems and applications, as well as the challenges associated with proteome libraries.
- 12 Walter JG, Kokpinar O, Friehs K, Stahl F, Schepet T. Systematic investigation of optimal aptamer immobilization for protein-microarray applications. *Anal. Chem.* 80(19), 7372–7378 (2008).
- 13 Renberg B, Nordin J, Merca A *et al.* Affibody molecules in protein capture microarrays: evaluation of multidomain ligands and different detection formats. *J. Proteome Res.* 6(1), 171–179 (2007).
- 14 Sanchez-Carbayo M. Antibody arrays: technical considerations and clinical applications in cancer. *Clin. Chem.* 52(9), 1651–1659 (2006).
- 15 Shafer MW, Mangold L, Partin AW, Haab BB. Antibody array profiling reveals serum TSP-1 as a marker to distinguish benign from malignant prostatic disease. *Prostate* 67(3), 255–267 (2007).
- 16 Robinson WH, DiGennaro C, Hueber W *et al.* Autoantigen microarrays for multiplex characterization of autoantibody responses. *Nat. Med.* 8(3), 295–301 (2002).
- 17 Chen S, Zheng T, Shortreed MR, Alexander C, Smith LM. Analysis of cell surface carbohydrate expression patterns in normal and tumorigenic human breast cell lines using lectin arrays. *Anal. Chem.* 79(15), 5698–5702 (2007).
- 18 Chan SM, Ermann J, Su L, Fathman CG, Utz PJ. Protein microarrays for multiplex analysis of signal transduction pathways. *Nat. Med.* 10(12), 1390–1396 (2004).
- 19 Ehrlich JR, Qin S, Liu BC. The ‘reverse capture’ autoantibody microarray: a native antigen-based platform for autoantibody profiling. *Nat. Protoc.* 1(1), 452–460 (2006).
- 20 Sheehan KM, Calvert VS, Kay EW *et al.* Use of reverse phase protein microarrays and reference standard development for molecular network analysis of metastatic ovarian carcinoma. *Mol. Cell Proteomics* 4(4), 346–355 (2005).
- 21 Grubb RL, Calvert VS, Wulkuhle JD *et al.* Signal pathway profiling of prostate cancer using reverse phase protein arrays. *Proteomics* 3(11), 2142–2146 (2003).
- 22 Akkiprik M, Nicorici D, Cogdell D *et al.* Dissection of signaling pathways in fourteen breast cancer cell lines using reverse-phase protein lysate microarray. *Technol. Cancer Res. Treat.* 5(6), 543–551 (2006).
- 23 Espina V, Liotta LA, Petricoin EF III. Reverse-phase protein microarrays for theranostics and patient tailored therapy. *Methods Mol. Biol.* 520, 89–105 (2009).
- 24 Bertone P, Snyder M. Advances in functional protein microarray technology. *FEBS J.* 272(21), 5400–5411 (2005).
- Highlights the developments in protein array technologies, such as printing and binding chemistry for generation of functional protein microarrays and their applications.
- 25 Issaq HJ, Chan KC, Janini GM, Conrads TP, Veenstra TD. Multidimensional separation of peptides for effective proteomic analysis. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 817(1), 35–47 (2005).
- 26 He M, Taussig MJ. Single step generation of protein arrays from DNA by cell-free expression and *in situ* immobilisation (PISA method). *Nucleic Acids Res.* 29(15), E73–E73 (2001).
- 27 Schwenk JM, Gry M, Rimini R, Uhlén M, Nilsson P. Antibody suspension bead arrays within serum proteomics. *J. Proteome Res.* 7(8), 3168–3179 (2008).
- 28 Wong J, Sibani S, Lokko NN, LaBaer J, Anderson KS. Rapid detection of antibodies in sera using multiplexed self-assembling bead arrays. *J. Immunol. Methods* 350(1–2), 171–182 (2009).
- 29 Elshal MF, McCoy JP. Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA. *Methods* 38(4), 317–323 (2006).
- 30 Nolan JP, Sklar LA. Suspension array technology: evolution of the flat-array paradigm. *Trends Biotechnol.* 20(1), 9–12 (2002).
- 31 Ramachandran N, Raphael JV, Hainsworth E *et al.* Next-generation high-density self-assembling functional protein arrays. *Nat. Methods* 5(6), 535–538 (2008).
- 32 Beare PA, Chen C, Bouman T *et al.* Candidate antigens for Q fever serodiagnosis revealed by immunoscreening of a *Coxiella burnetii* protein microarray. *Clin. Vaccine Immunol.* 15(12), 1771–1779 (2008).
- 33 Lopez JE, Beare PA, Heinzen RA *et al.* High-throughput identification of T-lymphocyte antigens from *Anaplasma* marginale expressed using *in vitro* transcription and translation. *J. Immunol. Methods* 332(1–2), 129–141 (2008).
- 34 Hurst R, Hook B, Slater MR, Hartnett J, Storts DR, Nath N. Protein–protein interaction studies on protein arrays: effect of detection strategies on signal-to-background ratios. *Anal. Biochem.* 392(1), 45–53 (2009).
- 35 Mei Q, Fredrickson CK, Jin S, Fan ZH. Toxin detection by a miniaturized *in vitro* protein expression array. *Anal. Chem.* 77(17), 5494–5500 (2005).
- 36 Meng L, Michaud GA, Merkel JS *et al.* Protein kinase substrate identification on functional protein arrays. *BMC Biotechnol.* 8, 22 (2008).
- 37 Ptacek J, Devgan G, Michaud G *et al.* Global analysis of protein phosphorylation in yeast. *Nature* 438(7068), 679–684 (2005).
- 38 Chen Z, Tabakman SM, Goodwin AP *et al.* Protein microarrays with carbon nanotubes as multicolor Raman labels. *Nat. Biotechnol.* 26(11), 1285–1292 (2008).
- Describes the innovative use of single-walled carbon nanotubes functionalized with multicolor Raman-labeled antibodies for detection of autoantibodies in Wegener’s granulomatosis.
- 39 Nam JM, Thaxton CS, Mirkin CA. Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins. *Science* 301(5641), 1884–1886 (2003).
- Describes an ultrasensitive bio-barcode detection technique for proteins using antibody-coated magnetic microparticles and DNA-bound nanoparticles.
- 40 Thaxton CS, Elghanian R, Thomas AD *et al.* Nanoparticle-based bio-barcode assay redefines ‘undetectable’ PSA and biochemical recurrence after radical prostatectomy. *Proc. Natl Acad. Sci. USA* 106(44), 18437–18442 (2009).
- 41 Edwards BS, Oprea T, Prossnitz ER, Sklar LA. Flow cytometry for high-throughput, high-content screening. *Curr. Opin. Chem. Biol.* 8(4), 392–398 (2004).
- 42 Gantelius J, Hartmann M, Schwenk JM, Roeraade J, Andersson-Svahn H, Joos TO. Magnetic bead-based detection of autoimmune responses using protein microarrays. *Nat. Biotechnol.* 26(6), 269–276 (2009).
- 43 Morozov VN, Morozova TY. Active bead-linked immunoassay on protein microarrays. *Anal. Chim. Acta* 564(1), 40–52 (2006).

- 44 Miller JC, Zhou H, Kwekel J *et al.* Antibody microarray profiling of human prostate cancer sera: antibody screening and identification of potential biomarkers. *Proteomics* 3(1), 56–63 (2003).
- 45 Haab BB. Methods and applications of antibody microarrays in cancer research. *Proteomics* 3(11), 2116–2122 (2003).
- 46 Srivastava M, Eidelman O, Jozwik C *et al.* Serum proteomic signature for cystic fibrosis using an antibody microarray platform. *Mol. Genet. Metab.* 87(4), 303–310 (2006).
- 47 Sreekumar A, Nyati MK, Varambally S *et al.* Profiling of cancer cells using protein microarrays: discovery of novel radiation-regulated proteins. *Cancer Res.* 61(20), 7585–7593 (2001).
- 48 Lin Y, Huang R, Santanam N, Liu YG, Parthasarathy S, Huang RP. Profiling of human cytokines in healthy individuals with vitamin E supplementation by antibody array. *Cancer Lett.* 187(1–2), 17–24 (2002).
- 49 Nielsen UB, Cardone MH, Sinskey AJ, MacBeath G, Sorger PK. Profiling receptor tyrosine kinase activation by using Ab microarrays. *Proc. Natl Acad. Sci. USA* 100(16), 9330–9335 (2003).
- 50 Schweitzer B, Kingsmore SF. Measuring proteins on microarrays. *Curr. Opin. Biotechnol.* 13(1), 14–19 (2002).
- 51 Shao W, Zhou Z, Laroche I *et al.* Optimization of rolling-circle amplified protein microarrays for multiplexed protein profiling. *J. Biomed. Biotechnol.* 2003(5), 299–307 (2003).
- 52 Zhou H, Bouwman K, Schotanus M *et al.* Two-color, rolling-circle amplification on antibody microarrays for sensitive, multiplexed serum-protein measurements. *Genome Biol.* 5(4), R28 (2004).
- 53 Han XX, Zhao B, Ozaki Y. Surface-enhanced Raman scattering for protein detection. *Anal. Bioanal. Chem.* 394(7), 1719–1727 (2009).
- 54 Li T, Guo L, Wang Z. Microarray based Raman spectroscopic detection with gold nanoparticle probes. *Biosens. Bioelectron.* 23(7), 1125–1130 (2008).
- 55 Cao YC, Jin R, Nam JM, Thaxton CS, Mirkin CA. Raman dye-labeled nanoparticle probes for proteins. *J. Am. Chem. Soc.* 125(48), 14676–14677 (2003).
- 56 Kim D, Daniel WL, Mirkin CA. Microarray-based multiplexed scanometric immunoassay for protein cancer markers using gold nanoparticle probes. *Anal. Chem.* 81(21), 9183–9187 (2009).
- 57 Liang RQ, Tan CY, Ruan KC. Colorimetric detection of protein microarrays based on nanogold probe coupled with silver enhancement. *J. Immunol. Methods* 285(2), 157–163 (2004).
- 58 Gao J, Liu D, Wang Z. Microarray-based study of carbohydrate-protein binding by gold nanoparticle probes. *Anal. Chem.* 80(22), 8822–8827 (2008).
- 59 Gao J, Liu C, Liu D, Wang Z, Dong S. Antibody microarray-based strategies for detection of bacteria by lectin-conjugated gold nanoparticle probes. *Talanta* 81(4–5), 1816–1820 (2010).
- 60 Resch-Genger U, Grabolle M, Cavaliere-Jaricot S, Nitschke R, Nann T. Quantum dots versus organic dyes as fluorescent labels. *Nat. Methods* 5(9), 763–775 (2008).
- 61 Sun YP, Zhou B, Lin Y *et al.* Quantum-sized carbon dots for bright and colorful photoluminescence. *J. Am. Chem. Soc.* 128(24), 7756–7757 (2006).
- 62 Jokerst JV, Raamanathan A, Christodoulides N *et al.* Nano-bio-chips for high performance multiplexed protein detection: determinations of cancer biomarkers in serum and saliva using quantum dot bioconjugate labels. *Biosens. Bioelectron.* 24(12), 3622–3629 (2009).
- 63 Hu M, Yan J, He Y *et al.* Ultrasensitive, multiplexed detection of cancer biomarkers directly in serum by using a quantum dot-based microfluidic protein chip. *ACS Nano*. 4(1), 488–494 (2010).
- Antibody-conjugated quantum dots were used for ultrasensitive multiplexed detection of serum cancer biomarkers with a femtomolar level of sensitivity and potential for direct use in serum.
- 64 Zajac A, Song D, Qian W, Zhukov T. Protein microarrays and quantum dot probes for early cancer detection. *Colloids Surf. B Biointerfaces* 58(2), 309–314 (2007).
- 65 Ghazani AA, Lee JA, Klostranec J *et al.* High throughput quantification of protein expression of cancer antigens in tissue microarray using quantum dot nanocrystals. *Nano Lett.* 6(12), 2881–2886 (2006).
- 66 Geho D, Lahar N, Gurnani P *et al.* Pegylated, streptavidin-conjugated quantum dots are effective detection elements for reverse-phase protein microarrays. *Bioconjug. Chem.* 16(3), 559–566 (2005).
- 67 Drbohlavova J, Adam V, Kizek R, Hubalek J. Quantum dots—characterization, preparation and usage in biological systems. *Int. J. Mol. Sci.* 10(2), 656–673 (2009).
- 68 Wu H, Huo Q, Varnum S *et al.* Dye-doped silica nanoparticle labels/protein microarray for detection of protein biomarkers. *Analyst* 133(11), 1550–1555 (2008).
- 69 Wang L, Yang C, Tan W. Dual-luminophore-doped silica nanoparticles for multiplexed signaling. *Nano Lett.* 5(1), 37–43 (2005).
- 70 Santra S, Zhang P, Wang K, Tapec R, Tan W. Conjugation of biomolecules with luminophore-doped silica nanoparticles for photostable biomarkers. *Anal. Chem.* 73(20), 4988–4993 (2001).
- 71 Deng T, Li JS, Jiang JH, Shen GL, Yu RQ. Preparation of near-IR fluorescent nanoparticles for fluorescence-anisotropy-based immunoagglutination assay in whole blood. *Adv. Funct. Mater.* 16(16), 2147–2155 (2006).
- 72 Goluch ED, Nam JM, Georgopoulou DG *et al.* A bio-barcode assay for on-chip attomolar-sensitivity protein detection. *Lab. Chip.* 6(10), 1293–1299 (2006).
- 73 Oh BK, Nam JM, Lee SW, Mirkin CA. A fluorophore-based bio-barcode amplification assay for proteins. *Small* 2(1), 103–108 (2006).
- 74 Stoeva SI, Lee JS, Smith JE, Rosen ST, Mirkin CA. Multiplexed detection of protein cancer markers with biobarcoded nanoparticle probes. *J. Am. Chem. Soc.* 128(26), 8378–8379 (2006).
- 75 Faucher S, Martel A, Sherring A *et al.* Protein bead array for the detection of HIV-1 antibodies from fresh plasma and dried-blood-spot specimens. *Clin. Chem.* 50(7), 1250–1253 (2004).
- 76 Morgan E, Varro R, Sepulveda H *et al.* Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. *Clin. Immunol.* 110(3), 252–266 (2004).
- 77 Sklar LA, Carter MB, Edwards BS. Flow cytometry for drug discovery, receptor pharmacology and high-throughput screening. *Curr. Opin. Pharmacol.* 7(5), 527–534 (2007).
- 78 Schwenk JM, Lindberg J, Sundberg M, Uhlen M, Nilsson P. Determination of binding specificities in highly multiplexed bead-based assays for antibody proteomics. *Mol. Cell Proteomics* 6(1), 125–132 (2007).
- 79 Leblanc N, Gantelius J, Schwenk JM *et al.* Development of a magnetic bead microarray for simultaneous and simple detection of four pestiviruses. *J. Virol. Methods* 155(1), 1–9 (2009).

- 80 Stahl PL, Gantelius J, Natanaelsson C, Ahmadian A, Andersson-Svahn H, Lundeberg J. Visual DNA – identification of DNA sequence variations by bead trapping. *Genomics* 90(6), 741–745 (2007).
- 81 Ramachandran N, Larson DN, Stark PR, Hainsworth E, Labaer J. Emerging tools for real-time label-free detection of interactions on functional protein microarrays. *FEBS J.* 272(21), 5412–5425 (2005).
- 82 Blow N. Proteins and proteomics: life on the surface. *Nat. Methods* 6, 389–393 (2009).
- 83 Zhu SL, Zhang JB, Lin Yue LY, Hartono D, Liu AQ. Label-free protein detection via gold nanoparticles and localized surface plasmon resonance. *Adv. Mat. Res.* 74, 95–98 (2009).
- 84 D'Urso OS, De Blasi MD, Manera MG, Latronico MF, Rella R, Poltronieri P. *Listeria monocytogenes* detection with surface plasmon resonance and protein arrays. *IEEE Sens. J.* 458–461 (2008).
- 85 Wassaf D, Kuang G, Kopacz K et al. High-throughput affinity ranking of antibodies using surface plasmon resonance microarrays. *Anal. Biochem.* 351(2), 241–253 (2006).
- 86 Klenkar G, Liedberg B. A microarray chip for label-free detection of narcotics. *Anal. Bioanal. Chem.* 391(5), 1679–1688 (2008).
- 87 Ladd J, Taylor AD, Piliarik M, Homola J, Jiang S. Label-free detection of cancer biomarker candidates using surface plasmon resonance imaging. *Anal. Bioanal. Chem.* 393(4), 1157–1163 (2009).
- 88 Lausted C, Hu Z, Hood L. Quantitative serum proteomics from surface plasmon resonance imaging. *Mol. Cell Proteomics* 7(12), 2464–2474 (2008).
- Application of surface plasmon resonance imaging for high-throughput, label-free profiling and quantification of serum from liver cancer patients using antibody microarrays.
- 89 Inoue Y, Mori T, Yamanouchi G et al. Surface plasmon resonance imaging measurements of caspase reactions on peptide microarrays. *Anal. Biochem.* 375(1), 147–149 (2008).
- 90 Ouellet E, Lausted C, Lin T, Yang CW, Hood L, Lagally ET. Parallel microfluidic surface plasmon resonance imaging arrays. *Lab. Chip.* 10(5), 581–588 (2010).
- 91 Srivastava S, Labaer J. Nanotubes light up protein arrays. *Nat. Biotechnol.* 26(11), 1244–1246 (2008).
- 92 Okuno J, Maehashi K, Kerman K, Takamura Y, Matsumoto K, Tamiya E. Label-free immunosensor for prostate-specific antigen based on single-walled carbon nanotube array-modified microelectrodes. *Biosens. Bioelectron.* 22(9–10), 2377–2381 (2007).
- 93 Joshi A, Punyani S, Bale SS, Yang H, Borca-Tasciuc T, Kane RS. Nanotube-assisted protein deactivation. *Nat. Nanotechnol.* 3(1), 41–45 (2008).
- 94 Welsher K, Liu Z, Sherlock SP et al. A route to brightly fluorescent carbon nanotubes for near-infrared imaging in mice. *Nat. Nanotechnol.* 4(11), 773–780 (2009).
- 95 Cid CC, Riu J, Maroto A, Rius FX. Carbon nanotube field effect transistors for the fast and selective detection of human immunoglobulin G. *Analyst* 133(8), 1005–1008 (2008).
- 96 Villamizar RA, Maroto A, Rius FX, Inza I, Figueras MJ. Fast detection of *Salmonella infantis* with carbon nanotube field effect transistors. *Biosens. Bioelectron.* 24(2), 279–283 (2008).
- 97 Zheng G, Patolsky F, Cui Y, Wang WU, Lieber CM. Multiplexed electrical detection of cancer markers with nanowire sensor arrays. *Nat. Biotechnol.* 23(10), 1294–1301 (2005).
- 98 Wang WU, Chen C, Lin KH, Fang Y, Lieber CM. Label-free detection of small-molecule-protein interactions by using nanowire nanosensors. *Proc. Natl Acad. Sci. USA* 102(9), 3208–3212 (2005).
- 99 Patolsky F, Zheng G, Hayden O, Lakadamyali M, Zhuang X, Lieber CM. Electrical detection of single viruses. *Proc. Natl Acad. Sci. USA* 101(39), 14017–14022 (2004).
- Novel application of nanowire field-effect transistors for detection of influenza and adenovirus particles using antibody-modified nanowire arrays.
- 100 Brammer KS, Choi C, Oh S et al. Antibiofouling, sustained antibiotic release by Si nanowire templates. *Nano Lett.* 9(10), 3570–3574 (2009).
- 101 Hwang KS, Lee S, Kim SK, Lee JH, Kim TS. Micro- and nanocantilever devices and systems for biomolecule detection. *Annu. Rev. Anal. Chem.* 2, 77–98 (2009).
- 102 Braun T, Ghatkesar MK, Backmann N et al. Quantitative time-resolved measurement of membrane protein-ligand interactions using microcantilever array sensors. *Nat. Nanotechnol.* 4(3), 179–185 (2009).
- Specific and quantitative detection of bacteriophage interaction with transmembrane receptors reconstituted in proteoliposomes by microcantilever array sensors.
- 103 Sarphie D. Nanomechanical cantilever biosensors: a springboard to novel antibiotics. *Innovations in Pharmaceutical Technology* 29, 12–17 (2009).
- 104 Wee KW, Kang GY, Park J et al. Novel electrical detection of label-free disease marker proteins using piezoresistive self-sensing micro-cantilevers. *Biosens. Bioelectron.* 20(10), 1932–1938 (2005).
- 105 Yue M, Stachowiak JC, Lin H, Datar R, Cote R, Majumdar A. Label-free protein recognition two-dimensional array using nanomechanical sensors. *Nano Lett.* 8(2), 520–524 (2008).
- 106 Braun T, Backmann N, Vogtli M et al. Conformational change of bacteriorhodopsin quantitatively monitored by microcantilever sensors. *Biophys. J.* 90(8), 2970–2977 (2006).
- 107 Huber F, Lang HP, Hegner M, Despont M, Drechsler U, Gerber C. Analyzing refractive index changes and differential bending in microcantilever arrays. *Rev. Sci. Instrum.* 79(8), 086110 (2008).
- 108 Dauksaitė V, Lorentzen M, Besenbacher F, Kjems J. Antibody-based protein detection using piezoresistive cantilever arrays. *Nanotechnology* 18(18), 125503 (2007).
- 109 Zhang H, Wu G, Tu H, Huang F. Discovery of serum biomarkers in astrocytoma by SELDI-TOF MS and proteinchip technology. *J. Neurooncol.* 84(3), 315–323 (2007).
- 110 Zhu XD, Zhang WH, Li CL, Xu Y, Liang WJ, Tien P. New serum biomarkers for detection of HBV-induced liver cirrhosis using SELDI protein chip technology. *World J. Gastroenterol.* 10(16), 2327–2329 (2004).
- 111 Grus FH, Podust VN, Bruns K et al. SELDI-TOF-MS ProteinChip array profiling of tears from patients with dry eye. *Invest. Ophthalmol. Vis. Sci.* 46(3), 863–876 (2005).
- 112 Cui JF, Liu YK, Zhou HJ et al. Screening serum hepatocellular carcinoma-associated proteins by SELDI-based protein spectrum analysis. *World J. Gastroenterol.* 14(8), 1257–1262 (2008).
- 113 Mannello F, Medda V, Toni GA. Protein profile analysis of the breast microenvironment to differentiate healthy women from breast cancer patients. *Expert Rev. Proteomics* 6(1), 43–60 (2009).

- 114 Liu Y, Wang H, Huang J, Yang J, Liu B, Yang P. Microchip-based ELISA strategy for the detection of low-level disease biomarker in serum. *Anal. Chim. Acta* 650(1), 77–82 (2009).
- 115 Breitenstein M, Holzel R, Bier FF. Immobilization of different biomolecules by atomic force microscopy. *J. Nanobiotechnology* 8, 10 (2010).
- 116 Soultani-Vigneron S, Dugas V, Rouillat MH *et al.* Immobilisation of oligo-peptidic probes for microarray implementation: characterisation by FTIR, atomic force microscopy and 2D fluorescence. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 822(1–2), 304–310 (2005).
- 117 Lynch M, Mosher C, Huff J, Nettikadan S, Johnson J, Henderson E. Functional protein nanoarrays for biomarker profiling. *Proteomics* 4(6), 1695–1702 (2004).
- 118 Lee M, Kang DK, Yang HK *et al.* Protein nanoarray on Prolinker surface constructed by atomic force microscopy dip-pen nanolithography for analysis of protein interaction. *Proteomics* 6(4), 1094–1103 (2006).
- 119 Lian W, Litherland SA, Badrane H *et al.* Ultrasensitive detection of biomolecules with fluorescent dye-doped nanoparticles. *Anal. Biochem.* 334(1), 135–144 (2004).
- 120 Lee W, Lee JH, Oh BK, Choi JW. Detection of human serum albumin on protein array using scanning tunneling microscopy. *Ultramicroscopy* 110(6), 723–728 (2010).
- 121 Kanga D, Janga Y, Leea J, Kimb S, Oha B, Choi J. Electrical detection of prostate specific antigen on protein array using scanning tunneling microscopy. *Curr. Appl. Phys.* 9(2), e33–e37 (2009).
- 122 Yang L, Li Y, Erf GF. Interdigitated array microelectrode-based electrochemical impedance immunosensor for detection of *Escherichia coli* O157:H7. *Anal. Chem.* 76(4), 1107–1113 (2004).
- 123 Xu H, Mao X, Zeng Q, Wang S, Kawde AN, Liu G. Aptamer-functionalized gold nanoparticles as probes in a dry-reagent strip biosensor for protein analysis. *Anal. Chem.* 81(2), 669–675 (2009).
- 124 Komarova E, Reber K, Aldissi M, Bogomolova A. New multispecific array as a tool for electrochemical impedance spectroscopy-based biosensing. *Biosens. Bioelectron.* 25(6), 1389–1394 (2010).
- 125 Nedelkov D, Nelson RW. Practical considerations in BIA/MS: optimizing the biosensor-mass spectrometry interface. *J. Mol. Recognit.* 13(3), 140–145 (2000).
- 126 Ozkumur E, Needham JW, Bergstein DA *et al.* Label-free and dynamic detection of biomolecular interactions for high-throughput microarray applications. *Proc. Natl Acad. Sci. USA* 105(23), 7988–7992 (2008).
- 127 Joo C, Ozkumur E, Unlu MS, Boer JF. Spectral-domain optical coherence phase microscopy for label-free multiplexed protein microarray assay. *Biosens. Bioelectron.* 25(2), 275–281 (2009).
- 128 Zhu H, Bilgin M, Bangham R *et al.* Global analysis of protein activities using proteome chips. *Science* 293(5537), 2101–2105 (2001).
- 129 Arenkov P, Kukhtin A, Gemmell A, Voloshchuk S, Chupeeva V, Mirzabekov A. Protein microchips: use for immunoassay and enzymatic reactions. *Anal. Biochem.* 278(2), 123–131 (2000).
- 130 Heijmans-Antoniissen C, Wesseldijk F, Munnikes RJ *et al.* Multiplex bead array assay for detection of 25 soluble cytokines in blister fluid of patients with complex regional pain syndrome type 1. *Mediators Inflamm.* 2006(1), 28398 (2006).
- 131 Khan SS, Smith MS, Reda D, Suffredini AF, McCoy JP Jr. Multiplex bead array assays for detection of soluble cytokines: comparisons of sensitivity and quantitative values among kits from multiple manufacturers. *Cytometry B. Clin. Cytom.* 61(1), 35–39 (2004).
- 132 Konry T, Hayman RB, Walt DR. Microsphere-based rolling circle amplification microarray for the detection of DNA and proteins in a single assay. *Anal. Chem.* 81(14), 5777–5782 (2009).
- 133 Hiep HM, Endo Y, Kerman K *et al.* A localized surface plasmon resonance based immunosensor for the detection of casein in milk. *Sci. Technol. Adv. Mater.* 8, 331 (2007).
- 134 Wu G, Datar RH, Hansen KH, Thundat T, Cote RJ, Majumdar A. Bioassay of prostate-specific antigen (PSA) using microcantilevers. *Nat. Biotechnol.* 19, 856–860 (2001).
- 135 Lee K, Kim E, Mirkin CA, Wolinsky SM. The use of nanoarrays for highly sensitive and selective detection of human immunodeficiency virus type 1 in plasma. *Nano Lett.* 4 (10), 1869–1872 (2004).
- 136 Vorderwülbecke S, Cleverley S, Weinberger SR, Wiesner A. Protein quantification by the SELDI-TOF-MS-based ProteinChip® System. *Nat. Methods* 2, 393–395 (2005).