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Label-Free Technologies for Quantitative Multiparameter Biological Analysis

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Abstract

In the post-genomic era, information is king and information-rich technologies are critically important drivers in both fundamental biology and medicine. It is now known that single-parameter measurements provide only limited detail and that quantitation of multiple biomolecular signatures can more fully illuminate complex biological function. Label-free technologies have recently attracted significant interest for sensitive and quantitative multiparameter analysis of biological systems. There are several different classes of label-free sensors that are currently being developed both in academia and in industry. In this critical review, we highlight, compare, and contrast some of the more promising approaches. We will describe the fundamental principles of these different methodologies and discuss advantages and disadvantages that might potentially help one in selecting the appropriate technology for a given bioanalytical application.

Keywords

Genomics/Proteomics; Bioassays; Biochips/High throughput screening; Biosensors; Clinical/Biomedical analysis; Immunoassays/ELISA

Introduction

High information content genomic and proteomic technologies, such as capillary sequencing, cDNA microarrays, 2-D poly(acrylamide) gel electrophoresis, and mass spectrometry, have greatly increased the level of molecular clarity with which we now understand human biology. Perhaps the most critical insight gleaned from these continued efforts is the vast interconnectivity of gene and protein regulatory networks. This in turn leads to the realization that biological systems are more completely characterized as an increasing number of molecular expression profiles are obtained from a single analysis. Coupled with immortalized cell lines and modern molecular and cell biology techniques, the aforementioned genomic and proteomic tools are well-suited and established in research laboratories. Unfortunately, many of the same measurement approaches are not rigorously quantitative and also are not ideal for use in the clinic where sample sizes and specialized training in analytical methods are more limited.

The greatest challenges in quantitative clinical bioanalysis arise due the requirement of a label —usually fluorescent or enzymatic. This label may be either directly tethered to the biomolecule under interrogation or alternatively to a secondary or tertiary recognition element such as in a sandwich assay configuration. In the case of antibody-based sandwich assays, such

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as conventional enzyme-linked immunosorbent assays (ELISAs), the requirement for a secondary protein capture agent adds significant cost and development time as generation of multiple, high binding affinity antibodies that recognize distinct and non-overlapping target epitopes can be very difficult. Direct labeling has its own challenges. Label incorporation itself can be highly heterogeneous, making any resulting measurement inherently non-quantitative [1]. Furthermore, Sun et al. recently demonstrated that the presence of a fluorescent label can have detrimental effects on the affinity of an antigen-antibody interaction [2]. Since almost all biosensing methods essentially provide a measure of surface receptor occupancy, which is dictated by the binding affinity between the capture agent and antigen, this report validates the quite obvious fact that labels can, in many cases, negatively impact the limit of detection of an assay.

For these reasons, among others, there is a great interest in developing label-free methods of biomolecular analysis. There are many different classes of label-free biosensors, but all are based upon the measurement of an inherent molecular property such as refractive index or mass. In this review, we focus on examples of label-free biosensors in which multiple target analytes are assayed simultaneously from within the same sample. These transduction methodologies, which are often based upon micro- and nanotechnologies, therefore also have an advantage of relatively low sample consumption, since multiple sample volumes are not required for multiple assays.

Clearly it would be impossible to discuss every technology that fits the label-free multiplexed sensing criteria, therefore, in this review we have attempted to highlight some of the technologies that we feel are the most promising at present to make an impact in this rapidly developing field. We have chosen to break the review down into sections according to the manner in which the presence of the biological moiety is transduced: plasmonic, photonic, electronic, and mechanical methods. In each section we briefly introduce the key aspects of sensor operation, highlight notable research to date, and comment on the advantages and/or disadvantages of each technology as it applies to the direct, multiplexed analysis of complex biological samples. In some cases we will discuss applicability to detection of different classes of biomolecules and/or cells. Furthermore, while this review is mainly focused on detection (quantitation) we will also highlight several instances where label-free techniques have been used in multiplexed molecular library (chemical and biological) screening applications, since many of these demonstrations are highly relevant and potentially amenable to multiparameter quantitation.

We have intentionally chosen not to discuss other valuable analytical metrics such as time-to-result and specificity. Our reasoning is that while critically important to sensor utility, these metrics can be severely complicated by other factors that are not related to the fundamental physical performance of the device. Properties such as the dimensions of the sample chamber surrounding the sensor [3,4] or quality of the capture agent can dominate observed time response and specificity (and sensitivity, for that matter). We recognize that these are vitally important facts to consider, but it is not practical to qualify each literature report in terms of the many peripheral factors that affect these performance attributes.

Plasmonic Methods

One promising technique for the multiplexed, label-free detection of biomolecules is surface plasmon resonance imaging (SPRI), also referred to as surface plasmon resonance microscopy (SPRM). SPRI is based upon the same fundamental principles as conventional surface plasmon resonance spectroscopy (SPR); light is coupled to the interface of a thin metallic film (typically gold for biosensing applications) via total internal reflection where propagating surface plasmon modes are excited, if the photons are of a particular frequency and incident angle. The

evanescent field associated with the plasmon resonance samples the proximal optical dielectric environment and is highly sensitive to local changes in refractive index, including those associated with the binding of biomolecules to receptors presented by the surface [5]. When biomolecules bind to specific receptors anchored to the metallic film, the corresponding changes in the refractive index modulate the intensity of light reflected off of the surface, which in turn is measured by the detector [6].

SPR was first demonstrated for biosensing applications by Lundstrom in 1983 [7], and was further developed throughout the mid-1980s as a method to monitor immunochemical reactions [8]. SPRI, which allows multiple binding events to be monitored simultaneously, was introduced by Yeatman and Ash in 1987 [9] and further developed by Corn and colleagues through and mid- to late-1990s [10–12]. In a typical SPRI experiment, shown schematically in Figure 1, a CCD detector is used to image the intensity of light reflected off of the surface, which directly corresponds to the amount of material bound to the metal film at a given image position. In this arrangement, changes in reflected light intensity can be measured down to a resolution of ~4 μm, allowing for highly multiplexed measurements of a variety of biological binding events [13]. However, multiplexing comes with a cost; SPRI typically has limits of detection 10–100 times higher (worse) that standard, non-imaging SPR [6].

One major application of SPRI is the readout of massive protein microarrays. Shumaker-Parry et al. demonstrated that the change in intensity of reflected light in an SPRI array could be correlated to a change in mass per unit area for proteins [14]. They successfully utilized this methodology to detect the binding of streptavidin to a biotinylated-DNA substrate, with a limit of detection of ~0.5 pg per 200 micron spot [15]. In later work, highlighted in Figure 2, the same group demonstrated quantitative measurement of the sequence-specific binding of the transcription factor Gal4 to double-stranded DNA sequences in a 120-component array with similar sensitivity [16].

An incredible breadth of SPRI applications in proteomics have been described over the past decade. This literature is far too broad to cover in its entirety here, and we direct those interested to two outstanding reviews on SPRI technologies for biomolecular interaction monitoring [5, 13]. Though many screening demonstrations have focused on only a limited number of components, SPRI systems are capable of higher levels of multiplexing—perhaps allowing for 10,000 or more parallel measurements [17].

There has also been significant effort focused on utilizing SPRI for quantitative nucleic acid analysis; work pioneered by the group of Prof. Robert Corn. Nelson and coworkers monitored hybridization of DNA and RNA onto microarrays at concentrations as low as 10 nM for short oligonucleotides (18-mers) and down to 2 nM using longer oligonucleotides (1500 bases) [18]. Goodrich et al. used an enzymatic approach to further extend the detection limit to ~1 fM [19,20]. These reports take advantage of the ability of RNase H to selectively cleave RNA from DNA:RNA heteroduplexes—allowing the rate of duplex hydrolysis to be correlated to the amount of bound target DNA. Lee et al. developed a related enzymatic amplification scheme using ExoIII, an enzyme that selectively cleaves DNA:DNA homoduplexes [21]. In this report, the authors were able to detect down to 10 pM of target DNA. Wolf et al. demonstrated the use of SPRI as a tool for screening small molecule-DNA interactions by observing the interaction between actinomycin-D with a multitude of DNA sequences [22]. While all of the aforementioned demonstrations of nucleic acid analysis used a limited number of array components, this technology clearly could be scaled to much higher levels of multiplexing.

Two variations to the traditional prism-based SPR techniques that have attracted considerable attention for multiplexed biosensing are grating-coupled SPR (GCSPR) and waveguide-coupled SPR (WCSPR). GCSPR utilizes an optical grating incorporated into the sensor surface

to generate high diffracted orders that couple photons to the surface and in turn launch propagating surface plasmons [23–25]. This technique provides a number of advantages to the tradition methods of SPR imaging. Prisms or index-matching fluids are not required for the generation of surface plasmons providing flexibility in the experimental layouts. Furthermore, the sensors can be mass produced with relative ease and low cost. However, the sensitivity of GCSPR is generally lower than that of prism-based SPR measurements [6]. WCSPR involves the incorporation of an optical fiber or other waveguide into the sensor as a means to generate surface plasmons. While the incorporation of a waveguide allows for miniaturization of the sensor, the coupling of the light from the waveguide to the surface plasmons is heavily dependent on the polarization of the incident light, which is sensitive to deformations in the waveguide geometry, thus limiting the general utility of the technique.

The widespread utility of SPRI as a biomolecular transduction technology is, in part, reflected in the number of companies offering commercial instrumentation. Biacore, owned by GE Healthcare, is the largest maker of SPRI instruments, offering a variety of models for different scales of analysis [26]. GWC Technologies, Inc. currently manufactures an SPRI instrument with the ability to screen over 25 different analytes simultaneously [27]. IBIS Technologies offers a versatile SPRI system that provides both fixed and scanning angle measurements for increased sensitivity [28]. Toyobo, manufacturer of the MultiSPRinter, packages a microarray spotter with their SPR imager, integrating the entire fabrication process within a single product [29]. GenOptics has developed commercial SPRI instruments capable of interrogating arrays having greater than 1,000 different components [30].

SPRI is a robust technology that has proven to be a valuable tool for the label-free detection and analyses of biomolecules. The technique possesses the ability to sensitively detect a wide range of biomolecules, including nucleic acids, proteins, and carbohydrates. On account of relatively large area of individual sensing elements, SPRI has a relatively poor limit of detection in terms of absolute bound mass, which might be a drawback in some sample-limited applications. While SPRI has not been widely utilized for multiparameter quantitation (examples focused almost exclusively on nucleic acid detection), the many successful demonstrations of multiplexed interaction screening make it a promising technology for such applications.

Another plasmonic-based biosensing platform that has recently emerged is based on the phenomenon of extraordinary optical transmission through periodic, subwavelength nanoholes in metallic thin films [31]. The intensity of light transmitted through these substrates is significantly higher than predicted by classical theory and has been shown to be mediated by surface plasmons [32]. The periodic holes act as a high-order diffraction grating that launches propagating plasmons linking the front- and back-sides of the metal thin film. The propagating plasmons then decouple from the substrate by emission of a new photon from the backside of the film [33]. Since propagating plasmons are sensitive to changes in the local refractive index, similar to SPR, nanohole arrays are responsive to biomolecular surface binding events. However, in this system the change is transduced as a shift in the wavelength of light maximally transmitted by the nanohole array. Several recent reports of nanohole-based biosensors have emerged out of Larson group [34,35]. The authors have rigorously defined the factors that effect device sensitivity and demonstrated the sensitive detection of anti-glutathione S-transferase antibodies down to concentrations as low as 10 nM on arrays that are capable of supporting at least 25 simultaneous measurements, as shown in Figure 3. Advantages of nanohole arrays for biomolecular detection include the extremely small footprint of the active sensing area (down to 1 μm^2) and the batch fabrication potential of the substrates, both of which should greatly facilitate high levels of sensor multiplexing (yet to be demonstrated).

Photonic Techniques

In addition to SPRI and nanohole arrays, several non-plasmonic optical biosensors currently show promise for high-throughput, multiparameter analysis—we term these photonic techniques. Photonic-based, label-free biosensing is not a new concept. As early as 1937, Langmuir and Schaefer described a method for evaluating the thickness of adsorbed monolayers of biomolecules on a metal surface by observing the colors generated by reflective interference [36]. In the late-1960s, Vroman and Adams demonstrated the use of ellipsometry for measuring immunoadsorbed molecules on a surface [37]. Following a similar timeline as SPR, optical biosensors based on technologies such as optical waveguides and reflective interferometry began to emerge in the 1990s [38,39]. Examples that we will discuss here include photonic crystals, optical microcavity resonators, reflective interferometry, and imaging ellipsometry. Typically, these methodologies take advantage of microscale fabrication methods and incorporate imaging or rapid scanning to interrogate a large number of sensors simultaneously or in near real-time.

Cunningham and co-workers[40] have pioneered a novel photonic crystal biosensing platform that has proven to be effective for multiplex screening and detection. Photonic crystals are engineered to selectively reflect a narrow bandwidth of light, and the wavelength at which this maximal reflection occurs is sensitive to the refractive index environment surrounding the substrate. As a result, bound biomolecules cause a measurable shift in the reflected wavelength (see Figure 4) [40,41]. The wavelength shift directly corresponds to the amount of bound biomolecule, and thus can be used for quantitation. Using this principle of detection, photonic crystal biosensors have demonstrated the ability to detect adsorbed protein down to ~1 pg/mm^2 on the surface [42].

For performing multiplexed measurements, plastic-molded photonic crystal structures have been incorporated into a microplate format for use with conventional biological assays, and multiple wells can be analyzed simultaneously using optical imaging techniques. Photonic crystal sensors are well-suited for multiparameter detection and quantitation of biological analytes [40,42,43]; however, many of the most relevant demonstrations to date have focused on multiplexed biomolecular interaction screening. For example, by monitoring the density of cells within a microplate well, Chan and co-workers have demonstrated the ability to rapidly screen compounds for effects on rates of cancer cell proliferation and apoptosis [44,45]. They have also recently shown that a microplate photonic crystal format can be used to screen thousands of compounds for their ability to inhibit specific protein-DNA interactions [46]. Choi et al. incorporated 11-different microfluidic channels onto a 96-well microplate system in order to allow parallel determination of relative binding affinities between protein A and seven different IgGs using a single photonic crystal substrate [47].

Photonic crystal biosensors have enabled rapid, label-free monitoring of protein binding and cell growth, demonstrating the utility of these sensors for high-throughput screening applications. Scalable manufacturing methods have facilitated the commercialization of this technology, as it is currently available from SRU Biosystems [48]. To date, photonic crystal biosensors have not been rigorously utilized for quantitative concentration determination in complex solutions; however, their promise for multiplexed detection points towards future applications in these areas.

Optical microcavity sensors of various geometries have also shown promise for highly sensitive label-free detection [49]. These sensors are based on the refractive index sensitivity of cavity modes supported by microfabricated waveguide structures that satisfy the constructive interference condition: $m\lambda = 2\pi n_{eff}$, where m is an integer value, λ is the wavelength of light, and n_{eff} is the effective refractive index that is sampled by the optical mode. The wavelength

at which resonance occurs is extremely narrow due to exacting fabrication of the optical cavity (high-Q cavities). Because the resonant wavelength is a function of the refractive index environment surrounding the optical cavity, sensing is accomplished by measuring the change in resonant wavelength in response to biomolecular binding to the microcavity surface. The narrow resonance bandwidth of high-Q microcavities, amongst other factors, helps make small shifts resolvable, which translates into low detection limits for biomolecular binding events.

Using microtoroidal resonators, Armani et al. demonstrated single-molecule detection of the cytokine interleukin-2 binding to an antibody-modified microcavity [50]. Suter et al.[51] utilized liquid-core optical rings resonators (LCORRs) to detect DNA at a surface density of 4 pg/mm^2, and Zhu et al. demonstrated virus detection with LCORRs at 2.3×10^{-3} pfu/mL [52]. White and co-workers also introduced a multiplexed LCORR array incorporating up to eight antiresonant reflecting optical waveguides (ARROWs) coupled to a glass capillary allowing interrogation of multiple optical cavities [53,54]. In addition to containing multiple sensing elements, the active resonator element, the capillary, integrates fluid handling into the sensing system.

Using silicon-on-insulator (SOI) microring resonators, Ramachandran et al. measured bacteria down to concentrations of 10^5 CFU/mL [55]. Mandal et al. demonstrated an optofluidic system based on microfabricated photonic crystal cavities that are coupled to a waveguide bus on a patterned silicon-on-insulator (SOI) substrate [56,57]. Although they have demonstrated a 20-component-capable chip, they have not yet performed actual biosensing with their system.

Work by De Vos et al.[58] and Ramachandran et al.[55] have illustrated the potential for using SOI microring resonators for multiplexed biosensors. Notably, standard CMOS-compatible semiconductor processing should allow many sensors to be integrated onto a single chip, as shown in Figure 5. Furthermore, fabrication of both waveguides and microcavities on the same SOI substrate may offer significant advantages in terms of baseline noise, compared to coupling via even the most efficient free-standing extruded optical fiber approaches [59]. Our own unpublished work has demonstrated that biomolecular binding to arrays of twenty-four, 30-micron diameter SOI microring resonators can be simultaneously monitored with a sensitivity to surface-bound protein of ~1 pg/mm^2 [60]. Given the very small total surface area of SOI microring structures, this corresponds to detection limits of less than 100 attograms of total bound protein.

Thus far, optical microcavity resonators have shown promise for highly sensitive, label-free biosensing. In addition, the small size of the microcavities makes these biosensors more sensitive to the absolute mass of surface-adsorbed biomolecules compared to techniques which use larger sensing elements or surface areas. Multiplexed sensing with optical microcavity resonators appears promising, and literature demonstrations are expected in the near future.

Other photonic detection techniques such as ellipsometry and reflective interferometry also are candidates for label-free multiplexed biosensing applications. In general, these techniques sensitively measure small changes in optical thickness on a surface. In 1995, Jin et al. first demonstrated that imaging ellipsometry could be used to measure arrays of adsorbed biomolecules on a surface [61]. The technique is identical to traditional ellipsometry—the measurement of polarization changes in light reflected off a surface—except that a CCD imaging detector is utilized to simultaneously interrogate thousands of discrete locations on a functionalized surface. Standard imaging ellipsometry can measure coatings of biomolecules ~0.1 nm thick (average optical thickness) on a surface [62]. Using oblique-incidence reflectivity difference (OI-RD) microscopes, Zhu et al. reported sensitivity down to optical thicknesses of ~0.01 nm for adsorbed protein on a surface [63] and Wang et al. reported a simple array-based multiplexed analysis of common proteins (BSA, HSA, IgG, and fibrinogen)

[64]. Subsequent examples have demonstrated 48-element arrays with the ability to simultaneously detect human IgG, fibrinogen, and five protein markers for Hepatitis [62]. Zhu et al. have also shown that OI-RD can be used to analyze large arrays having hundreds of sensing elements [63,65]. However these spots were largely redundant, containing only one or two unique types of capture probes such as antibodies against IgG and albumin or nucleic acid arrays presenting only complements to a single DNA target sequence.

Currently, imaging ellipsometry allows measurement of large arrays of biomolecular interactions on a single surface with sensitivity comparable to SPR imaging [66,67]. Recent applications of the technique have shown the ability of imaging ellipsometry to detect multiple biomolecules simultaneously from complex samples. Although not as fully developed as SPRI, recent refinements and improvements making the technique more “user friendly” (see review by Jin [62]) may lead to an increased presence of the technique in multiparameter analyses.

Reflective interferometric platforms have also shown the ability to measure small changes in optical thickness of a biomolecular layer on a surface. Rather than measuring changes in polarization, as in the case of ellipsometry, reflective interferometric techniques utilize optical interference between incident photons reflecting off a thin film. Changes in reflectance can thus be correlated with differential interference that occurs as biomolecules attach to a reflective surface. The sensitivity of reflective-interferometric techniques enables measurement of changes in surface thickness of about 1 pm, corresponding to ~1 pg/mm^2 of adsorbed biomolecule on the surface [68,69]. Gauglitz et al. have applied reflective interferometric spectroscopy (RIfS) to the multiplexed measurement of biomolecules in 96- and 384-well plates [70–72]. Using a back-lit configuration with CCD imaging, the authors were able to perform real-time analyses of biological binding events. This technology has been demonstrated for multiplexed assays aimed at screening antibodies against triazine libraries (four antibodies against 36 different compounds) [71] as well as for epitope mapping of the enzyme transglutamase [72]. The same group has also utilized RIfS for measuring nucleic acid duplex melting [73] and cell binding [74]. A commercial RIfS platform is currently being developed by Biametrics [75].

Özkumur et al. have demonstrated a spectral reflectance imaging biosensor that measures small changes in the height of surface-adsorbed molecules due to interference caused by differences in optical pathlength [69]. Using a CCD camera, 200 different spots can be measured simultaneously allowing real-time kinetic measurements to be made by monitoring the binding regions through a glass microfluidic substrate. To date, they have demonstrated the interaction of a protein with a surface-bound capture agent at a detection limit of 19 ng/mL. A commercialized version of this technology is being developed by Zoiray Technologies, Inc. [76].

Zhao et al. reported a similar reflectometric system for immunosensing applications called the biological compact disc (commercialized by Quadraspec, Inc. [77]) [78]. This technique has been expanded for parallel analyses and is referred to as molecular interferometric imaging (MI2). MI2 has been used to measure prostate specific antigen (PSA) as well as interleukin-5 (IL-5) with detection limits of 60 pg/mL and 50 pg/mL, respectively, on a surface presenting 128 functionalized areas [79,80]. Currently, however, MI2 has only demonstrated the ability to measure the concentrations of two different proteins simultaneously. Mace et al. recently utilized an array-based sensor for measuring three different cytokines simultaneously and demonstrated a limit of detection of 25 pg/mL for interferon-γ; however, their sensitivity and reproducibility was limited by large variation in spot morphology as a result of needing a dry surface for analysis [81].

Although widely variable in experimental setup (front- versus back-illuminated, fluid handling, etc.), reflective interferometric techniques have proven to be extremely sensitive to biomolecules adsorbed on a surface. Quantitative multiplexed assays based upon reflectometric techniques are still at an early stage of development, but molecular library screening applications are more mature. Future efforts in multiplexed analyses will likely lead to improvements in limits of detection for proteins and broaden the classes of target analytes to which the technology can be applied.

Electrical detection

Electrical detection methods have been suggested as attractive alternatives to optical readouts due to their low cost, low power consumption, ease of miniaturization, and potential multiplexing capability [82,83]. The basis of these detection systems stem from a binding-induced change in some electrical property of the circuit, of which the sensor is a vital component. Electronic biosensing platforms that we will discuss here include semiconducting silicon nanowires (SiNWs), carbon nanotubes (CNTs), and electrochemical impedance spectroscopy (EIS).

The most common mode of biomolecular detection using semiconductor nanowires is based on the principles of the field effect transistor (FET). In normal transistor operation, a semiconducting element is attached to a source and a drain electrode, and current flowing through the element is modulated by changing the voltage applied to a gate electrode [84]. In a FET-based nanowire biosensor configuration, the SiNW, functionalized with appropriate receptors such as ssDNA oligomers or antibodies, is connected to a source and drain electrode. Binding of target biomolecules changes the dielectric environment around the nanowires and plays a similar role as the gate electrode. Thus molecular binding can be directly quantitated as a change in the conductivity of the nanowires (see Figure 6) [85]. Compared to earlier planar FET sensors [86], the small size of nanowires means that individual binding events result in a more significant change in the electrical properties of the circuit. This unique feature of nanowire FETs provides ultrahigh sensitivity down to the single molecule level [87]. Since the first report of biosensing applications [88], silicon nanowires have been shown to be broadly applicable to a wealth of analytical challenges including the label-free detection of ions [88], small molecules [89], proteins [88,90,91], nucleic acids [92–94], viruses [87], and neuronal signals [95].

The first example of a multiplexed array of SiNW sensors was reported in 2004 by Patolsky et al. [87]. Surfaces of two p-type SiNWs were modified with monoclonal antibody receptors specific for *influenza A* and adenovirus particles, and selective binding and unbinding responses for each virus were detected in parallel at the single particle level. In 2005, Zheng et al. demonstrated the multiplexed detection of three cancer marker proteins, f-PSA, carcinoembryonic antigen (CEA), and mucin-1, in undiluted serum at femtomolar concentrations [90]. Notably, in this report the serum sample was desalted in order to reduce the solution ionic strength.

Nanowire sensing technology holds much promise for multiparameter biological sensing; however, there are some significant challenges to be addressed before routine operation of higher order multiplex analyses can be realized. One challenge that is inherent to FET-based detection systems is that they suffer reduced sensitivity when operated at physiological ionic strengths (~0.15 M). Ions in solution gate the FET similarly to the biomolecular target, and thus the device can experience a much diminished response to the binding event [96]. This can be avoided by desalting the sample prior to analysis, but requires the addition of a preparative step prior to analysis [84,94]. Though only partially related to the absolute mass sensitivity of

an individual nano-FET, the nanometer size regime of these sensing elements also makes their response highly dependent upon sample cell geometry and related parameters [3,4].

A second significant challenge relates to the integration of nanowires on substrates with reproducibility and uniformity [97]. Most reports of SiNW biosensors to date use nanowires fabricated via the vapor-liquid-solid (VLS) method, which gives high yields of uniform nanowires that have very advantageous properties for electronic and bioelectronic applications. Large numbers of VLS nanowires are grown simultaneously from individual catalyst particles and subsequent positioning and alignment on a sensor surface represents a significant hurdle. Recently, Fan et al. introduced a novel method that addresses this challenge achieving ~95% directional alignment of VLS nanowires via contact printing [97], which may greatly help facilitate integration of high density VLS nanowire sensing arrays.

An alternative method of preparing SiNW arrays was reported—originally for non-analytical applications —by Melosh et al. in 2003 [98]. This technique, termed superlattice nanowire pattern transfer (SNAP), utilizes a novel template and shadow mask approach to create ultrahigh density arrays of precisely aligned SiNWs on standard silicon-on-insulator (SOI) substrates. Using these SNAP nanowires, shown in Figure 7, Bunimovich and co-workers demonstrated label-free detection of sub-nanomolar (~100 pM) DNA concentrations [99]. Stern et al. have used CMOS-compatible SiNWs, though fabricated at considerably lower densities, for the detection of proteins (antibodies) below 100 fM [91]. A combination of the described scalable fabrication methods and ultrasensitive device operation may provide an attractive method for measuring the concentrations of many different biomolecules simultaneously.

Carbon nanotube (CNT)-based devices have also been widely investigated as biosensors due to their unique structure-dependent electronic and mechanical properties [100]. In particular, single-walled CNTs (SWNTs) can be metallic, semiconducting, or semi-metallic depending on the tube diameter and the chirality [101–103]. As a result, they have been used in a wide range of applications including, but not limited to, the FET transduction principle. CNT-based biosensors have been demonstrated for detection of small molecules [104,105], DNA (~50 nM) [103,106,107], Hepatitis C viral RNA (0.5 pM) [108], and immunoglobulin E (250 pM) [109]. Multiparameter detection of biomolecules using a FET operation modality has yet to be realized, but reports of multiplexed gas detection point towards the possibility [110]. Using AC voltammetry and multiwalled CNT arrays, Koehne et al.[107] demonstrated a readily scalable approach to DNA detection. While only applied to single parameter detection at moderate sensitivity (~100 nM), the authors describe a novel electrochemical etching and surface passivation scheme that should allow multiplexing. CNT arrays have shown potential for biological detection; however, widespread utility has been limited to date by difficulties in controlling the physical parameters relevant to biosensing: length, diameter, and chirality [101]. These issues are particularly significant for multiplexed sensing, in which uniform and reproducible performance of each sensor element is essential.

Another electrical transduction methodology that has shown promise for multiparameter biological detection is electrochemical impedance spectroscopy (EIS). In EIS, sensing is accomplished by measuring changes in the resistance and/or capacitance of the electrode-solution interface upon binding of a target molecule to a receptor-functionalized surface [82, 83]. Compared to other electrical measurements, such as amperometry and voltammetry, EIS does not require the use of enzymes to amplify binding events by generating Faradaic read-out signals. This is very significant because sensor crosstalk due to diffusion of enzymatic products would be a fatal problem for multiplexed detection applications [111]. Improvements to basic EIS operation have been reported that utilize alternative electrode materials such as polymers [112,113] and nanoparticles [114,115]. Furthermore, electrode geometry has been shown to

be extremely important, with arrays of interdigitated electrodes providing greater device sensitivity [83,116,117]. Various types of biological species have been detected using EIS including nucleic acids [116,118,119] down to 10 fM [120] and bacteria [117,121] as low as 10 cfu/mL [122]. Impedance-based sensing systems have also been applied to monitor protein-carbohydrate [123] and protein-protein [124,125] interactions. Recently, a demonstration of multiplexed protein interaction monitoring was reported by Yu et al. [111] where an array of gold electrodes was used to probe for four antibodies that recognized proteins immobilized on the electrodes.

At this stage, EIS appears to be promising for multiplexed biosensing and several commercialized systems are already available for cell-based screening [126] (from ACEA Bioscience [127] and Applied BioPhysics [128]). In these systems, cells grown in electrode-containing wells can be assayed for proliferation [129], adhesion and spreading [130,131], and cytotoxicity [129,131–133]. As the technology continues to mature, it will be necessary to develop a greater understanding of the exact mechanisms underlying the binding-induced change in impedance. This insight will then allow for better *a priori* design of experimental conditions and circuit modeling and fitting of the resulting data [83].

Mechanical sensors

Mechanical sensors are another promising tool for the multiplexed, label-free detection of biomolecules. Like all other label-free sensing methodologies, the specificity of these sensors is determined by the top-most coating layer, which can range from a modified gold surface to a variety of polymers [134–136]. One key advantage of this class of sensors is that it is amenable to a wide range of surface coatings.

Well-known acoustic wave biosensors, including quartz crystal microbalance (QCM) and integrated surface acoustic wave (SAW) technologies, are based on mechanical transduction and thus do not require labels. Both approaches utilize a piezoelectric quartz crystal connected to an oscillating external circuit that is able to measure the resonant oscillatory frequency of the system. Binding of molecules to the surface of the sensor are measured as shifts in the resonance frequency of the device [137]. These sensors have been utilized to detect a wide range of biomolecules, including nucleic acids [138–140], proteins [141–143], and lipids [144]. While extremely sensitive towards binding events, there are a number of factors that limit the effective utilization of acoustic wave devices for quantitative, multiplexed biosensing [145,146]. Most importantly, considerations such as viscoelasticity and hydration lead to non-linearities in frequency shifts accompanying biomolecular binding to the QCM surface [147–149].

A second class of mechanical sensors that has recently attracted considerable attention as a multiplexed, label-free biosensing platform is microcantilevers, as highlighted in Figure 8. Binding events on the cantilever surface are transduced via one of three methods: deflection of the cantilever [150], a change in the resonance frequency of the cantilevers [151], or a change in the stress exerted on the cantilever that generates an electric current in an attached piezoelectric element [136].

The simplest transduction event to monitor is the change in deflection, measured by reflecting a laser beam off the back of a cantilever and measuring position with a split photodiode. The binding of an analyte to the surface of the cantilever exerts a torque, meaning that the location on the cantilever at which the molecule binds affects the amount of deflection. Because each molecule does not generate the same amount of deflection, the position of bound molecules must be considered during deflection measurements (particularly important for large molecules). Furthermore, the required number of molecules bound in order to a surface to exert

a detectable deflection is significantly higher than with the other two transduction methods [152] (discussed below).

Owing to its ease of implementation, a wide range of biological molecules and even entire microorganisms have been have been detected using cantilever deflection, illustrated schematically in Figure 9. In 2000, Fritz et al. demonstrated the detection of a single base-pair mismatch within a 12-mer sequence of DNA [153]. Following this, McKendry and coworkers demonstrated the detection of DNA to concentrations as low as 75 nM with an 8-component array [154]. A number of protein systems have also been studied with this technique [155–157]. Of special interest is the work by Wu et al., in which prostate-specific antigen (PSA) was detected at clinically relevant levels as low as 0.2 ng/mL in serum containing 1 mg/mL albumin and plasminogen [158]. Recently, an interesting report out of the same lab demonstrated sensitive protein detection using very large arrays of up to 960 individually-readable microcantilevers [159]. Notably, though only a single protein was detected, PSA, the sensitivity was quite good, 1 ng/mL, and the sensor array was shown to have minimal response to non-specific proteins at much higher concentration.

Resonance-based transduction of microcantilevers involves monitoring the change in the resonance frequency upon binding of an analyte [151]. This is typically accomplished using one of a number of interferometric schemes. While the read-out equipment for this methodology is significantly more complex than for deflection-based methods, it is by far the most sensitive [150]. One limitation of resonance-based sensing is that the oscillations of microcantilevers are highly susceptible to dampening effects in liquids, which vary with solution composition.

Using the more sensitive resonance-based measurement strategy, involving the measurement of cantilever resonance frequency shifts, Ilic et al. demonstrated the detection of a *single strand* of DNA 1587 bp in length, having a mass of 1.7 attograms [160]. A number of protein systems have also been explored, with detection limits as low as 10 pg/mL for PSA [161, 162]. Studies focusing on the detection of microbes have also pushed the limits of detection down to single cells and virus particles [163–165].

The incorporation of piezoelectric materials into microcantilevers can also be used to probe the presence of biomolecules [136]. In the piezoelectric method, the binding of an analyte causes the cantilever to deflect, subsequently depolarizing the material and generating a current. It should also be noted that piezoelectric-modified microcantilevers can be used for both deflection and resonance-based measurement. While the piezoelectric method is less sensitive than the resonance method, it does not require the extensive optical layouts used in the other techniques and allows for incorporation of additional electronic components in the sensor. Even though detection systems have been demonstrated utilizing the piezoelectric read-out methodology, including examples of nucleic acid (LOD: 10 nM for a 12-mer) and protein (LOD: 5 ng/mL) analysis [166–168], significant advances are still needed to lower the limits of detection in order to compete with resonance-based methods.

Microcantilever-based sensors currently represent an attractive method for sensitive, label-free detection of biomolecules. The detection limits are comparable if not better than SPRI and the flexibility of operation and ease with which the cantilevers can be functionalized allows for virtually any system to be studied. Currently, several companies are developing commercial microcantilever-based systems, including Cantion [169], BioScale [170], and Concentris [171]. The advances stemming from both industry and several academic groups are rapidly advancing microcantilever technology for multiparameter bioanalytical applications.

Conclusions and Outlook

Taking values from a selection of literature sources and discussions with experts in the respective fields, we have compiled a table comparing many of the label-free biosensing technologies discussed in this review (Table 1), highlighting reported limits of detection, degree of multiplexing demonstrated in the literature, and status of commercialization. This is not meant to be a stand alone selection guide, as the specific requirements of the assay(s) should play a critical role in which technology best suits the application at hand. For example, absolute mass sensitivity may be of extreme importance in sample-limited applications and therefore a smaller surface area sensor, provided no loss in "bulk" sensitivity, might be advantageous. Another application might require very immediate analysis because of sample degradation, for example. In this situation, nanowire sensors requiring sample desalting might not be the best choice. Having small sensing features may allow for construction of higher density sensor arrays for more highly multiplexed applications. However, a significant discrepancy may exist between theoretical and "functional" sensor densities, which may be limited as much (or more) by the method of sensor derivitization rather than the dimensions of the sensing elements themselves. While it is certain that each technology will have specific advantages and disadvantages for a given application, each of the modalities described may be best option for a targeted multiparameter bioanalysis situation.

There are more specific applications and many additional compelling reasons that motivate the development of new and improvement of current label-free multiparameter biodetection technologies. The next several years promise to be an exciting time in this rapidly advancing field, which is poised to impact clinical diagnosis and disease management in the very near future.

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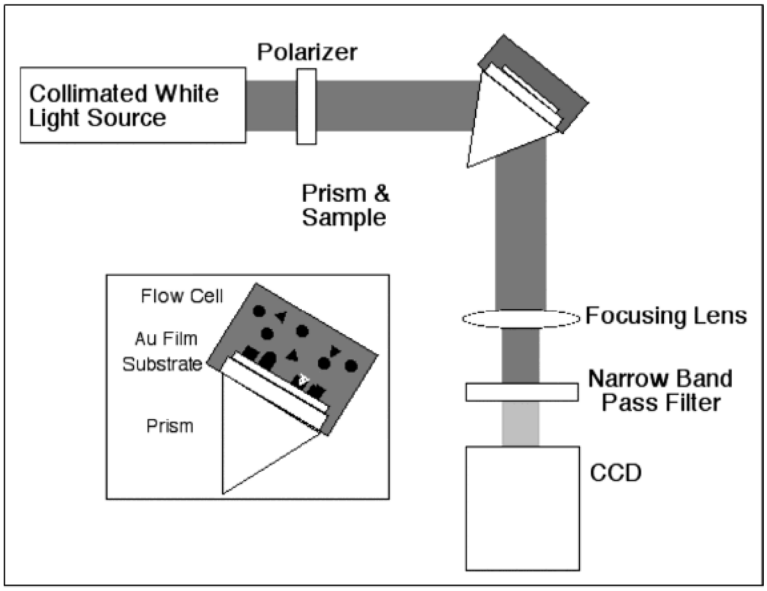

Figure 1.
Schematic representation of an imaging surface plasmon resonance (SPRI) instrumental configuration. Biomolecular binding events are transduced as a change in reflected light intensity, and multiplexing is accomplished by imaging a large portion of the substrate using the CCD. (Figure adapted from reference [5].)

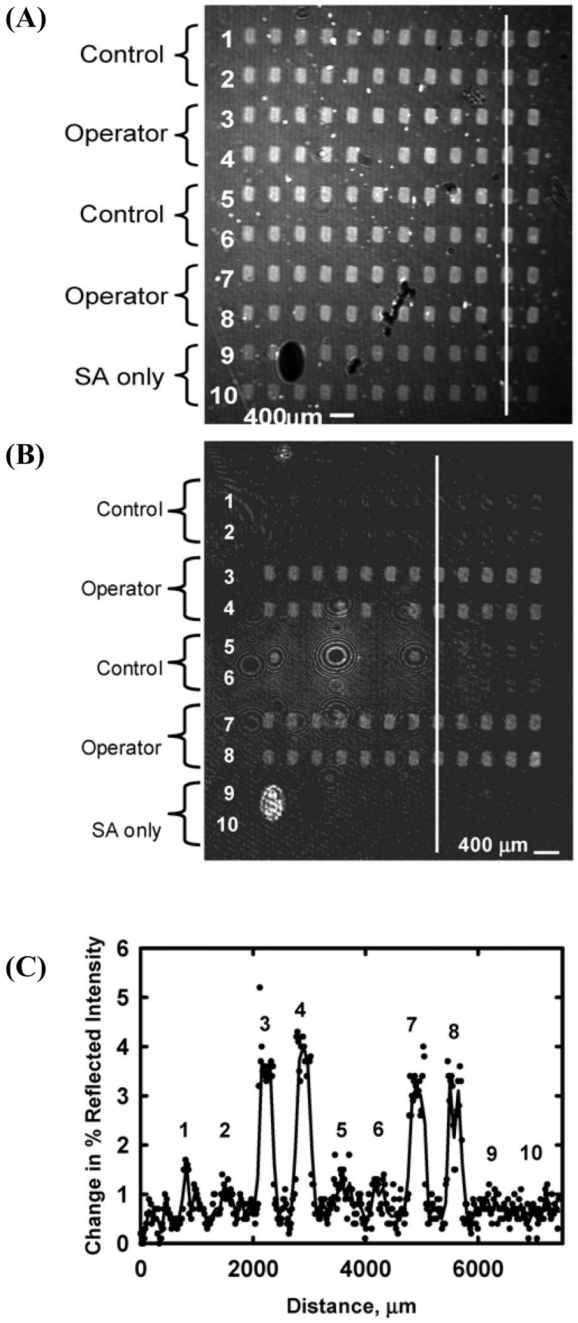

Figure 2.

(A) SPRI image of a 120-element dsDNA array. **(B)** Difference image and line scan **(C)** after incubation of array from (A) with the transcription factor Gal4. Specific protein binding is observed as a positive change in the reflected light image. (Figure adapted from reference [16].)

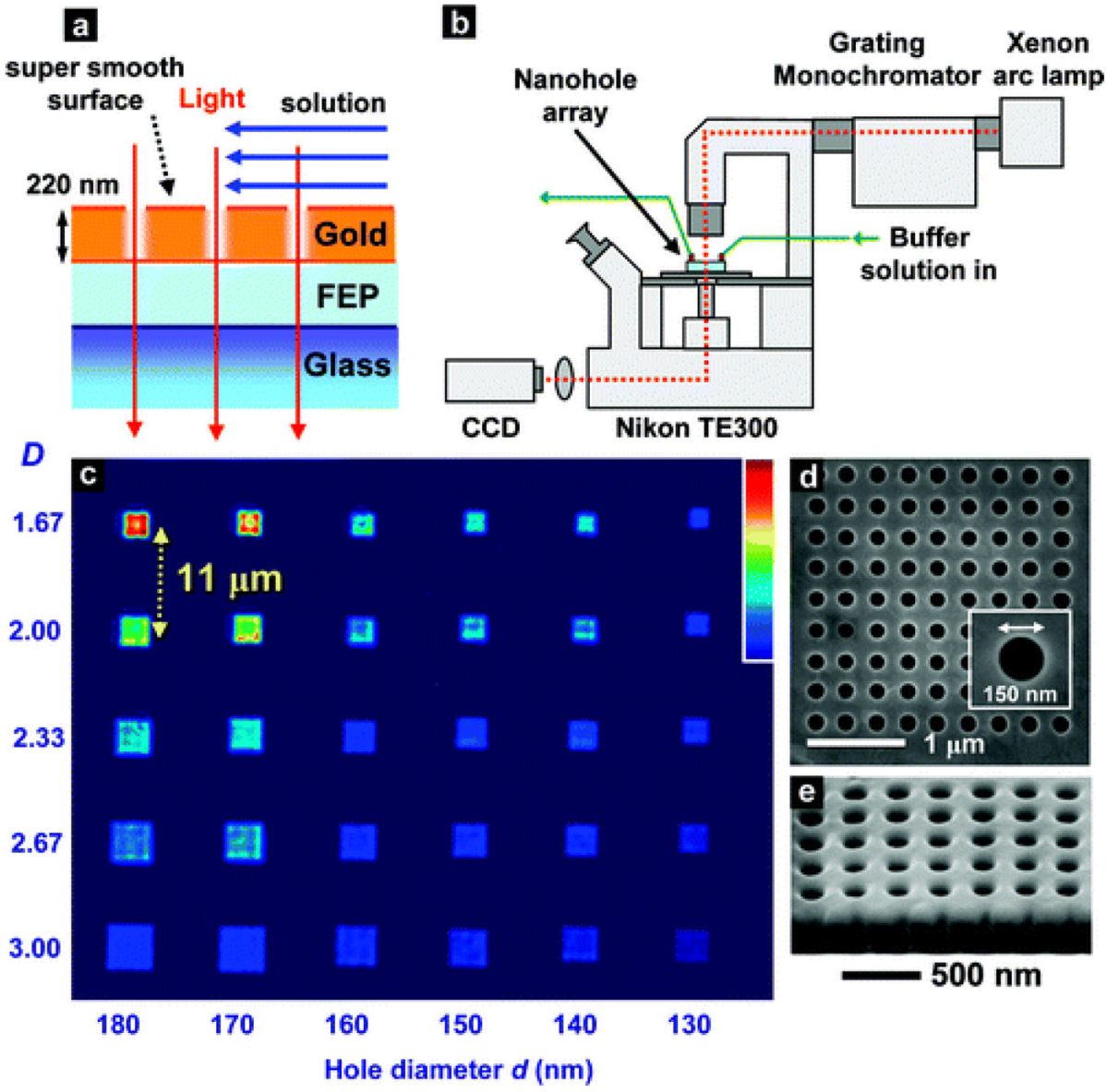

Figure 3.

(A) Schematic illustration of sample introduction onto a nanohole array biosensor. **(B)** Diagram showing nanohole array instrumental set up. **(C)** CCD image of 30 sets of nanohole arrays having different geometries. **(D, E)** Scanning electron micrographs showing two a top and side view of a 9 × 9 nanohole array. (Figure adapted from reference [34].)

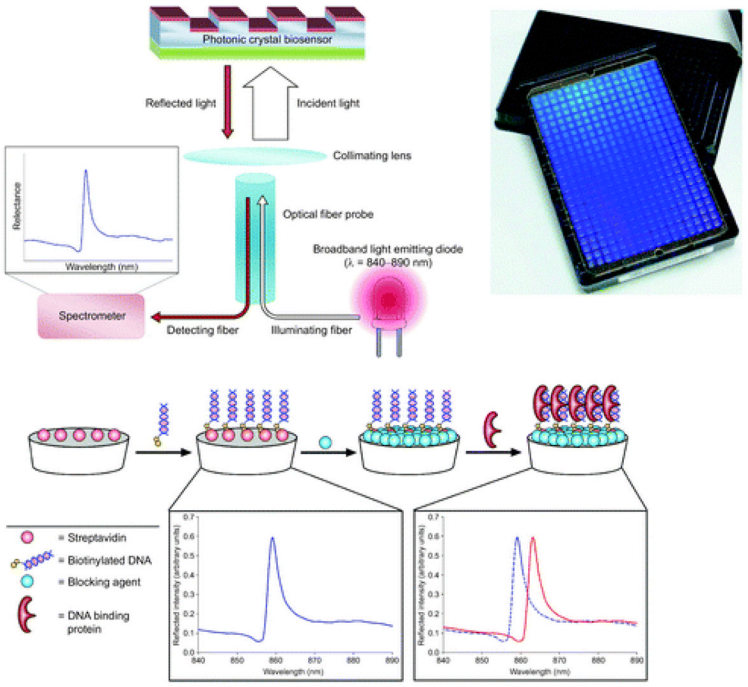

Figure 4.

Photonic crystal biosensors transduce biomolecular binding events by measuring the shift in wavelength of light reflected by the substrate. Shown here is a 384-well plate configuration of a photonic crystal sensing platform, which can be interrogated using a light emitting diode and simple spectrometer. This example demonstrates the screening small molecule libraries for inhibiting a specific DNA-protein binding event. (Figure adapted from reference [46].)

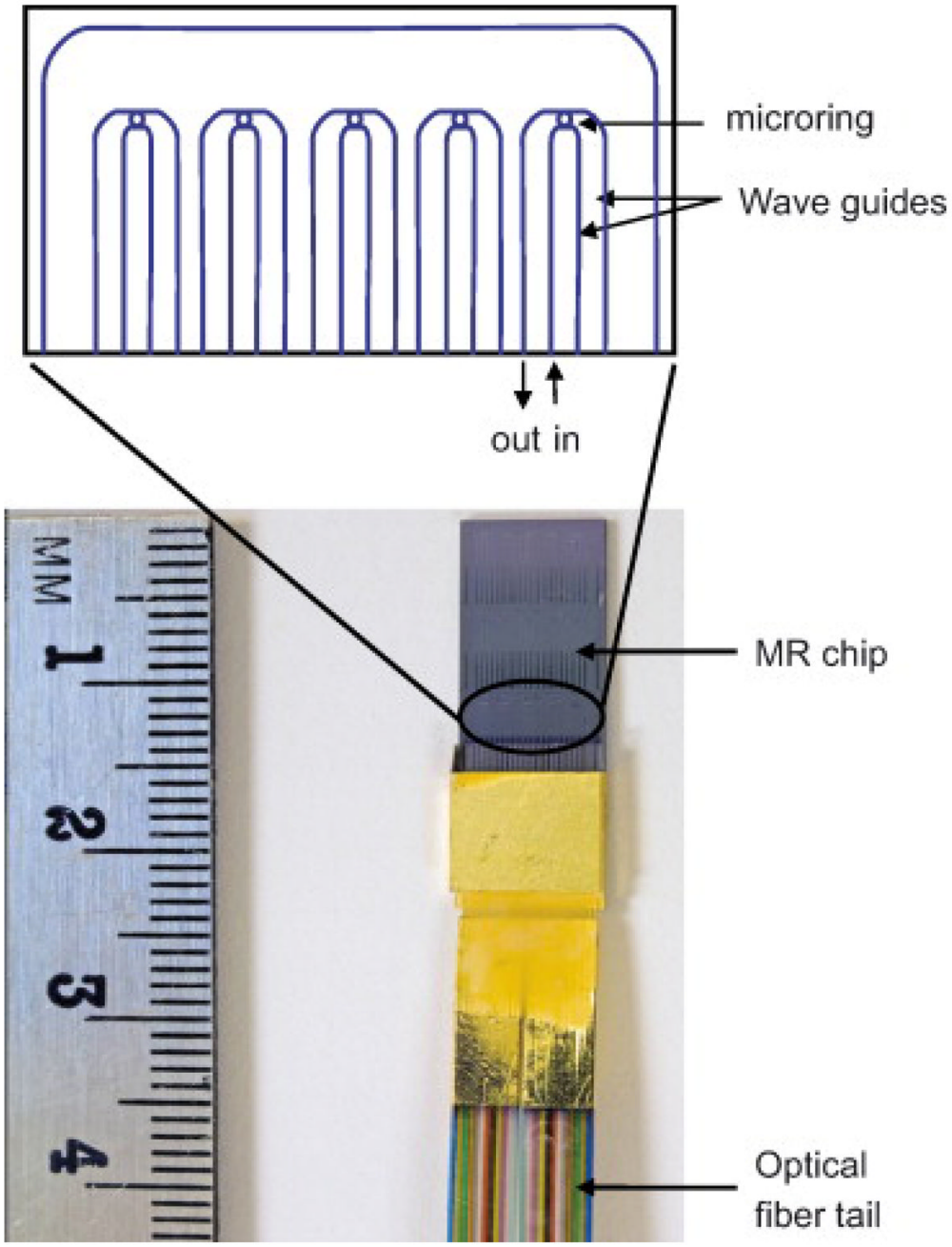

Figure 5.
Photograph of a five-ringed silicon-on-insulator microring resonator array used to detect biological binding events. In this example, the microrings are accessed by on-chip waveguides that are tapered off-chip to conventional fiber optics. (Figure from reference[55].)

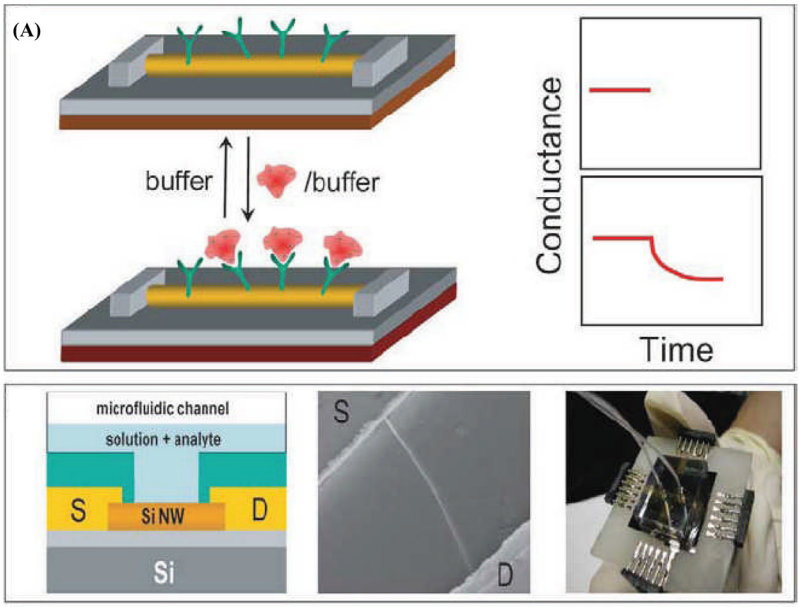

Figure 6.

(A) Schematic of a Si nanowire-based FET device configured as a sensor with antibody receptors (green), where binding of a protein with net positive charge (red) yields a decrease in the conductance. **(B)** Cross-sectional diagram and scanning electron microscopy image of a single Si nanowire sensor device, grown via the VLS method and a photograph of a prototype nanowire sensor biochip with integrated microfluidic sample delivery. (Figure adapted from reference [85].)

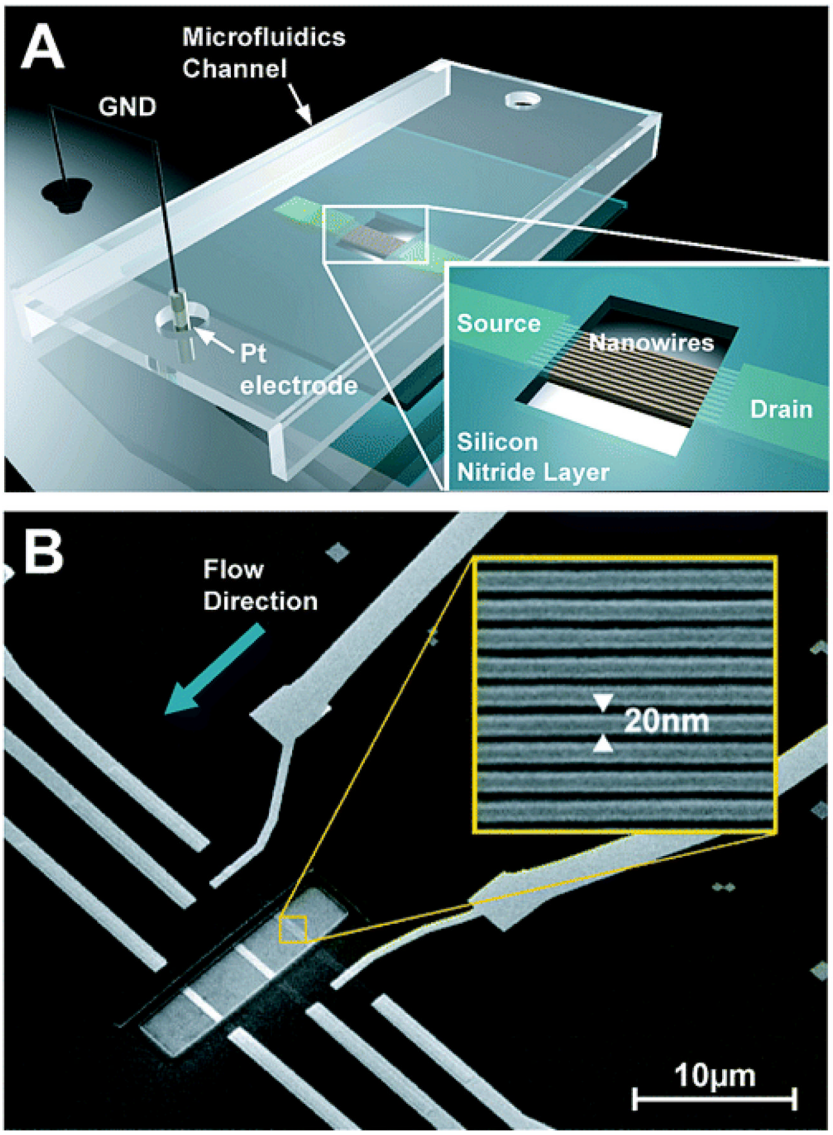

Figure 7.

A diagram **(A)** and scanning electron micrograph **(B)** of three groups of ten, 20-nm wide silicon nanowires used for label-free DNA detection. Using the superlattice nanowire patterning scheme, large numbers of precisely aligned nanowires can be fabricated for use as biosensors. (Figure from reference [99].)

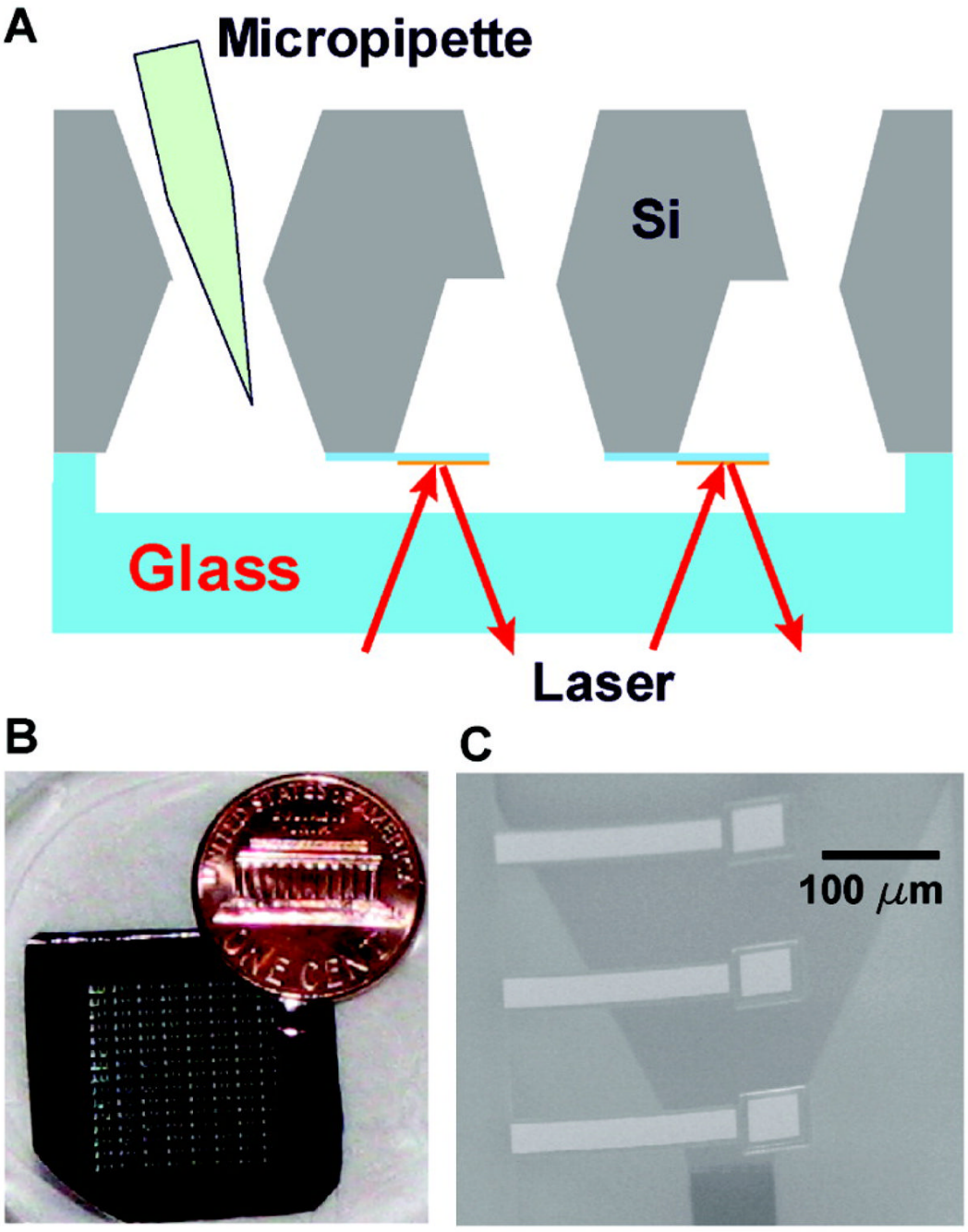

Figure 8.
Two-dimensional microcantilever array chip used to monitor protein-protein interactions. **(A)** Schematic of a reaction well. There were multiple cantilevers in each reaction well. Laser light reflected off a cantilever's end pad was used to monitor the deflection of cantilevers. **(B)** A chip soaked in DI water. **(C)** A scanning electron micrograph of 3 cantilevers in a reaction well. (Figure adapted from reference [159].)

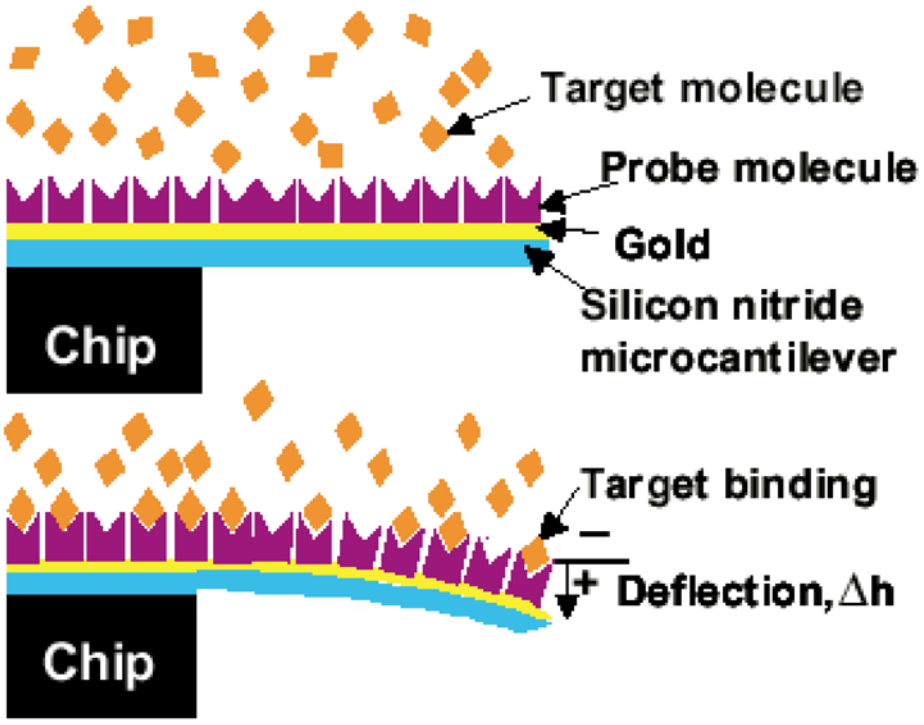

Figure 9.
A schematic showing the principle of deflection-based microcantilever biosensing. (Figure from reference [158].)

Table 1

Comparison of several promising label-free, multiparameter biosensing technologies.*

		Reported limit of detection (lowest values found in literature)	Multiplexing	Commercial Product?*
Plasmonic	SPRI	~1 fM for nucleic acids [19,20]; 0.5 pg per element for proteins [15,16]	1000+ elements demonstrated [17]	Yes [26–30]
	Nanohole Arrays	10 nM for proteins [35]	25 measurements demonstrated [35]	No
Photonic	Photonic Crystals	1 pg/mm^2 [40]	1536-well plate assays [48]	Yes [48]
	High-Q Microcavities	Microtoroids: Single-molecule (zeptogram) [50] SOI microrings: 80 attograms [60]	Two-component[172], larger arrays possible	No
	Imaging Ellipsometry	<1 ng/mL for protein [62]	~10 component assays demonstrated [62]	No
	Reflective Interferometry	1 pg/mm^2 [68,69]	384-well plate assays [70]	Yes [76,77]
Electronic	Silicon Nanowires	10 fM for DNA [92,173]; ~3pM for protein [174]	3 parameters demonstrated [174]	No
	Carbon Nanotubes	0.5 pM for RNA strand [108]; 250 pM for a protein [175]	Single parameter demonstrated	Yes [176]
	EIS	~25 pM for protein [125]	Four proteins detected[177]	Yes [127,128]
Mechanical	Microcantilevers	1.7 attograms [160]	320+ demonstrated [159]	Yes [169–171]

*Compiled to the best of the authors' knowledge at the time of submission. In addition to our own expertise and searching, additional information was sought from experts in the respective fields. The authors apologize for any unintentional oversights.