Microplate-based label-free detection of biomolecular interactions: applications in protemics

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This review describes a new type of label-free optical biosensor that is inexpensively manufactured from continuous sheets of plastic film and incorporated into standard format microplates to enable highly sensitive, high-throughput detection of small molecules, proteins and cells. The biosensor and associated detection instrumentation are applied to review two fundamental limiting issues for assays in proteomics research and drug discovery: requirement for quantitative measurement of protein concentration and specific activity, and measurements made with complex systems in highly parallel measurements. SRU Biosystems, Inc.'s BIND™ label-free detection will address these issues using data examples for hybridoma screening, epitope binning and mapping, small-molecule screening, and cell-based functional assays. The review describes several additional applications that are under development for the system, and the key issues that will drive adoption of the technology over the next 5 years.

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Limitations of label-based assay methods

The vast majority of assays currently used in pharmaceutical screening utilize some type of label to enable quantization of protein, DNA, small molecules, cells or the interactions of these entities. Typical labeling methods include the use of fluorophores, radioligands and secondary reporters. In contrast with the large variety of labeled methods, there are relatively few methods that enable detection of molecular and cellular interactions without labels. Label-free detection removes experimental uncertainty induced by the effect of the label on molecular conformation, blocking of active binding epitopes, steric hindrance, inaccessibility of the labeling site or the inability to find an appropriate label that functions equivalently for all molecules in an experiment [1]. The labeled approach also has the significant limitation of only reporting on the progress of an experiment when the labeled reagent is added to the reaction, and not on any other materials used. This limitation seriously disrupts quantification methods (e.g., concentration, activity and affinity) and data comparisons from experiment to experiment. The problems are further amplified when attempting to specifically identify molecules within complex pools of biological samples, which is required for proteomics research. Label-free detection methods greatly simplify the time and effort required for assay development and provide quantitative analysis, while removing experimental artifacts from quenching, shelf life and background fluorescence [2].

Label-free assays

Label-free detection generally involves the use of a transducer that is capable of directly measuring some physical property of a chemical compound, DNA molecule, peptide, protein or cell. For example, all biochemical molecules and cells have finite mass, volume, viscoelasticity, dielectric permittivity and conductivity that can be used to indicate their presence or absence using an appropriate

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sensor. The sensor functions as a transducer that can convert one of these physical properties (such as the mass of a substance deposited on the sensor's active surface) into a quantifiable signal that can be gathered by an appropriate instrument (e.g., a current or voltage that is proportional to the deposited mass).

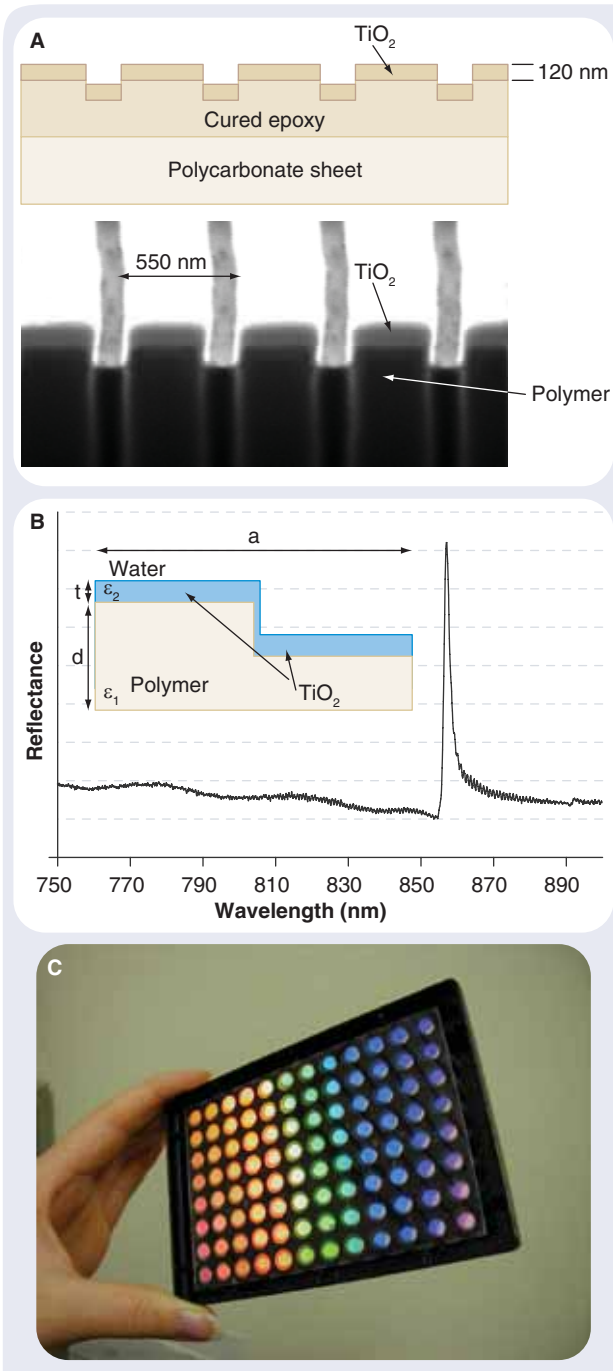

Figure 1. (A) Photonic crystal biosensor structure utilizing a 1D periodic surface structure of low dielectric constant polymer and high dielectric constant coating of TiO_2. Due to its simple structure, the sensor can be fabricated on continuous sheets of plastic film. (B) When illuminated with white light, a narrow band reflectance spectrum is obtained whose peak wavelength is tuned by the adsorption of biochemical material on the sensor surface. (C) A 96-well microplate incorporating the photonic crystal biosensor into the entire bottom surface of each of the wells.

Optical biosensors are designed to produce a measurable change in some characteristic of light that is coupled to the sensor surface. The advantage of this approach is that a direct physical connection between the excitation source (the source of illumination of the sensor), the detection transducer (a device that gathers reflected or transmitted light) and the transducer surface itself is not required. devising systems for interfacing the sensor with fluid exposure methods becomes greatly simplified when there is no need for electrical connections to the biosensor and no associated complications of packaging. Rather than detecting mass directly, all optical biosensors rely on the dielectric permittivity of detected substances to produce a measurable signal [3].

Adoption of a biosensor technology for most applications in diagnostics or pharmaceutical screening will be driven, to some extent, by the cost of performing an individual assay. A primary screen used in the pharmaceutical research industry (e.g., a screening campaign to determine a set of candidate chemicals that have a desired affinity level for a protein) can involve over 1 million assays. Researchers working on high-volume industrialized assays describe the need to minimize the cost per data point in such a campaign. While optical biosensors offer tremendous advantage over labeled assay technologies by not requiring the use of tag reagents, the cost of the transducer used in each assay must be low enough to be used economically on a wide scale. This cost goal is an extreme challenge to the wide acceptance of optical biosensors, which are often high-precision optical components fabricated from expensive materials (such as glass, silicon or optical fiber) using highly exacting processes, such as photolithography, dielectric or metal deposition, and plasma etching. Even if a sensor is inexpensive to fabricate, the cost of packaging and testing must also be efficient. Therefore, for widest acceptance, an optical biosensor technology must be designed to be compatible with mass-production methods using inexpensive materials, such as plastics, so they can be used once before disposal.

The throughput of a sensor system will also determine its usefulness in large pharmaceutical screening campaigns, or for diagnostic tests in which a test sample must be measured for the contents of many different proteins. A biosensor embedded within a flow chamber will have a throughput that is limited by the number of parallel flow channels, and the time required to flush reagents away from a previous assay, to regenerate the sensor surface and to introduce a new test sample. Cuvette-based systems will have limited throughput due to the number of cuvettes that can be operated in parallel. For example, cuvettes can provide much greater parallelism than a flow cell-based system if the cuvette is a well within a standard 96-, 384- or 1536-well microplate. Flow across the sensor surface may help to overcome some mixing and surface transport limitations of the system, but may also compromise sensitivity and aforementioned economic factors (time and cost).

The ability to perform high-sensitivity detection of biochemical interactions in a format that concurrently provides high throughput and low cost per assay would enable label-free optical biosensor technology to address applications that have not previously been feasible on a commercial basis. This review presents a biomolecular interaction detection (BIND™) system based on an approach that utilizes a photonic crystal biosensor that is manufactured from continuous sheets of plastic film, and incorporated into standard 96-, 384- and 1536-well microplate formats for compatibility with standard robotic liquid-handling systems used in screening applications. Many currently available robotic liquid-handling systems are capable of sufficient mixing to provide the desired equilibrium binding conditions for the microtiter plate-based sensor format described here. The system utilizes single-use disposable biosensor labware and a simple, robust microplate reader instrument that is configured for compatibility with robotic microplate handlers. An advanced microplate reader with high-resolution imaging capability has also been developed for high-resolution label-free imaging of biomolecule or cell density distributions. The operation of the biosensor, sensor manufacturing and the design of the readout instrument will be described later. Results for using the BIND systems for hybridoma antibody screening, epitope binning and mapping, small-molecule screening, and cell protein expression are presented, which address critical issues presently found in proteomics experiments.

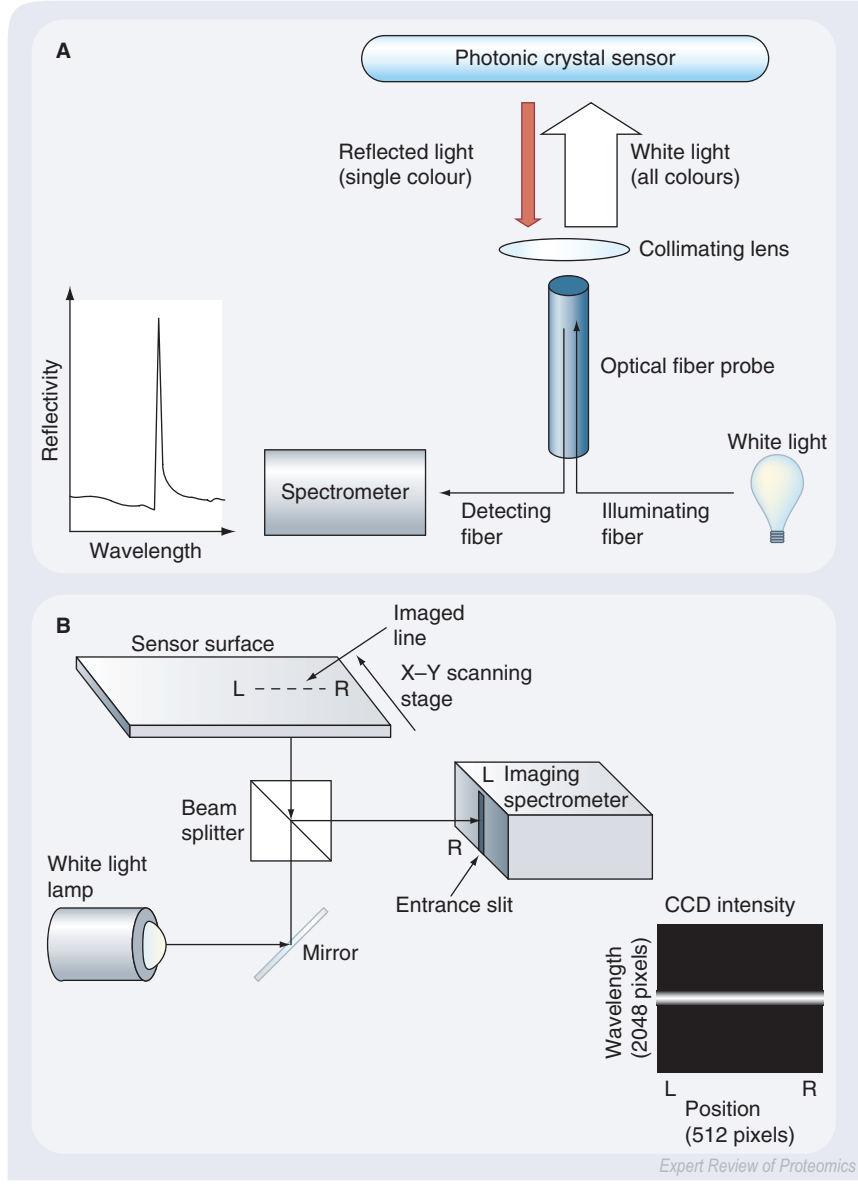

Figure 2. Excitation/detection instrumentation methods for photonic crystal biosensors.
(A) A light bulb illuminates the crystal surface at normal incidence through a fiber probe, and the reflected spectrum is gathered by a second fiber, connected to a spectrometer. High-resolution images of biochemical binding on the photonic crystal surface can be obtained using the instrument shown in (B), where an imaging spectrometer gathers hundreds of reflected spectra simultaneously from one line across the sensor surface.
CCD: Charge-coupled device; L: Left; R: Right.

Photonic crystal label-free biosensor

A new class of optical biosensors based on the unique properties of optical device structures, termed photonic crystals, have been recently developed [4,5]. A photonic crystal is composed of a periodic arrangement of dielectric material in two or three dimensions [6,7]. If the periodicity and symmetry of the crystal and the dielectric constants of the materials used are chosen appropriately, the photonic crystal will selectively couple energy at particular wavelengths, while excluding others [8]. The applications of structures such as these are numerous, including integration with lasers to inhibit or enhance spontaneous emission, waveguide angle-steering devices, and as narrowband optical filters [9–13]. Photonic crystal-structure geometry can be designed to concentrate light into extremely small volumes and to obtain very high local electromagnetic field intensities.

In order to adapt a photonic crystal device to perform as a biosensor, some portion of the structure must be in contact with a liquid test sample. By attaching biomolecules or cells to the portion of the photonic crystal where the locally confined electromagnetic field intensity is greatest, the resonant coupling of light into the crystal is modified, and thus the reflected/transmitted output is tuned. The highly confined

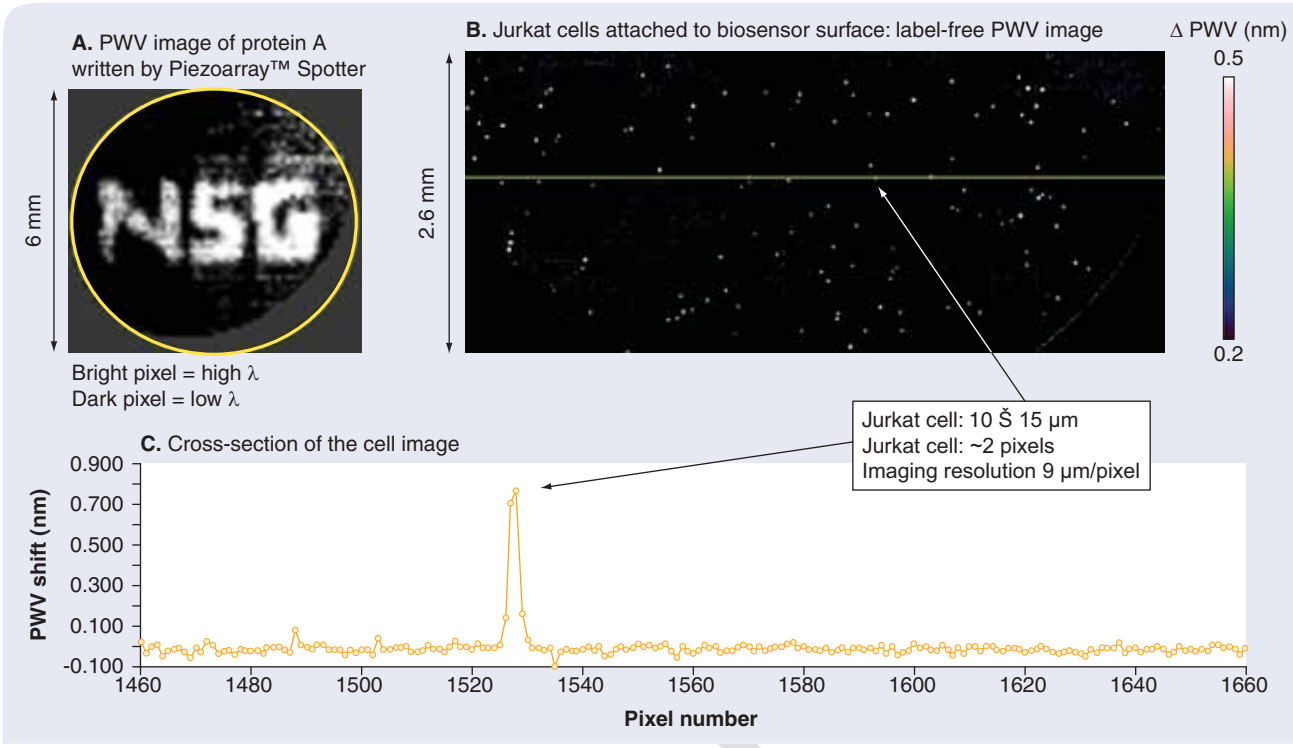

Figure 3. PWV shift images (bright regions represent regions of greater shift) of a photonic crystal sensor gathered using the instrument shown in FIGURE 2B. (A) A 6-mm diameter region of a biosensor is imaged at approximately 20-mm pixel resolution after writing the letters 'NSG' with a PerkinElmer, Inc. Piezoarray™ microarray spotting tool. (B) The instrument is used to image an approximately 2.5 × 7.0-mm region of the biosensor surface at 9-mm pixel resolution to record the localized ΔPWV caused by the attachment of individual cells. (C) The cells themselves are typically 10-15 mm in diameter, so they often overlap two adjacent pixels.
ΔPWV: PWV shift; PWV: Peak wavelength value.

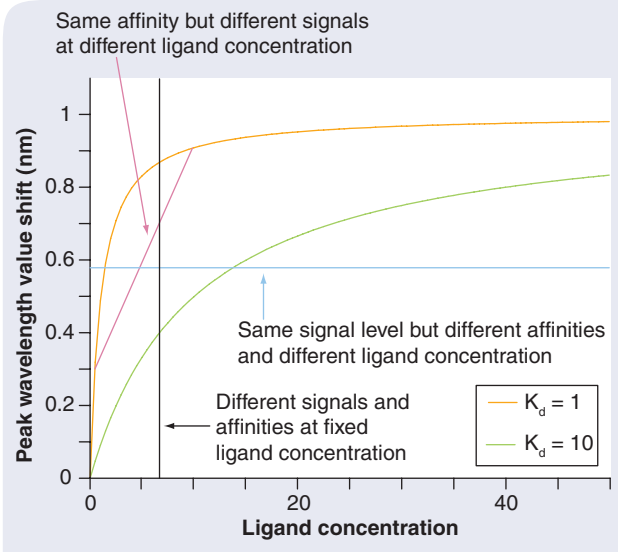

Figure 4. Description of relatedness of two antibodies or proteins with different affinities. An antibody of weak affinity (green curve), but present at higher concentration, can have a much higher signal than an antibody of high affinity (orange curve) that is present at a lower concentration. Notice where the three straight lines intersect the two curves, which represent simulations of binding curves for ligands with different affinities.
K_d: .

electromagnetic field within a photonic crystal structure provides high sensitivity and a high degree of spatial resolution consistent with their use in imaging applications, much like fluorescent imaging scanners.

Photonic crystal structures have their historical roots in a phenomenon termed Wood's anomaly. Wood's anomalies are effects observed in the spectrum of light reflected by optical diffraction gratings [14]. They manifest themselves as rapid variations in the intensity of particular diffracted orders in certain narrow frequency bands. They were first discovered by Wood in 1902 during some of the first experiments on reflection gratings, and were termed anomalies because the effects could not be explained by ordinary grating theory. Since that time, optical theory and numerical methods have developed so that structures making use of similar optical effects could be engineered to produce useful devices in the fields of telecommunications and optical displays [15]. For example, subwavelength periodic structures have been developed to reflect only a very narrow band of wavelengths when illuminated with white light [16]. To create a biosensor, a photonic crystal may be optimized to provide an extremely narrow resonant mode whose wavelength is particularly sensitive to modulations induced by the deposition of biochemical material on its surface [4]. A sensor structure, illustrated in FIGURE 1, consists of a low refractive index plastic material with a periodic surface structure that is over-coated

with a thin layer of high-refractive dielectric material. Device structures based on linear gratings and 2D gratings (i.e., arrays of holes, posts, or veins arranged in checkerboard or hexagonal close-packed grids along the sensor surface) have been demonstrated. The sensor is measured by illuminating the surface with white light, and collecting the reflected light with a noncontact optical fiber probe, where several parallel probes can be used to independently measure different locations on the sensor. The biosensor design enables a simple manufacturing process to produce sensor sheets in continuous rolls of plastic film that are hundreds of meters in length [17]. The mass manufacturing of a biosensor structure that is measurable in a noncontact mode over large areas enables the sensor to be incorporated into single-use disposable consumable items, such as 96-, 384- and 1536-well standard microplates, thereby making the sensor compatible with standard fluid-handling infrastructure employed in most laboratories.

The sensor operates by measuring changes in the wavelength of reflected light as biochemical binding events take place on the surface. For example, when a protein is immobilized on the sensor surface, an increase in the reflected wavelength is measured when a complementary binding protein is exposed to the sensor. Using low-cost components, the readout instrument is able to resolve protein mass changes on the surface with resolution less than 1 pg/mm^2. While this level of resolution is sufficient for measuring small-molecule interactions with immobilized proteins, the dynamic range of the sensor is also large enough to measure larger biochemical entities, including live cells, cell membranes, viruses and bacteria. A sensor measurement requires approximately 20 msec, and thus large numbers of interactions can be measured in parallel, and kinetic information can be gathered. The reflected wavelength of the sensor can be measured either in single-point mode (e.g., for measuring a single interaction within a microplate), or an imaging system can be used to generate an image of a sensor surface with less

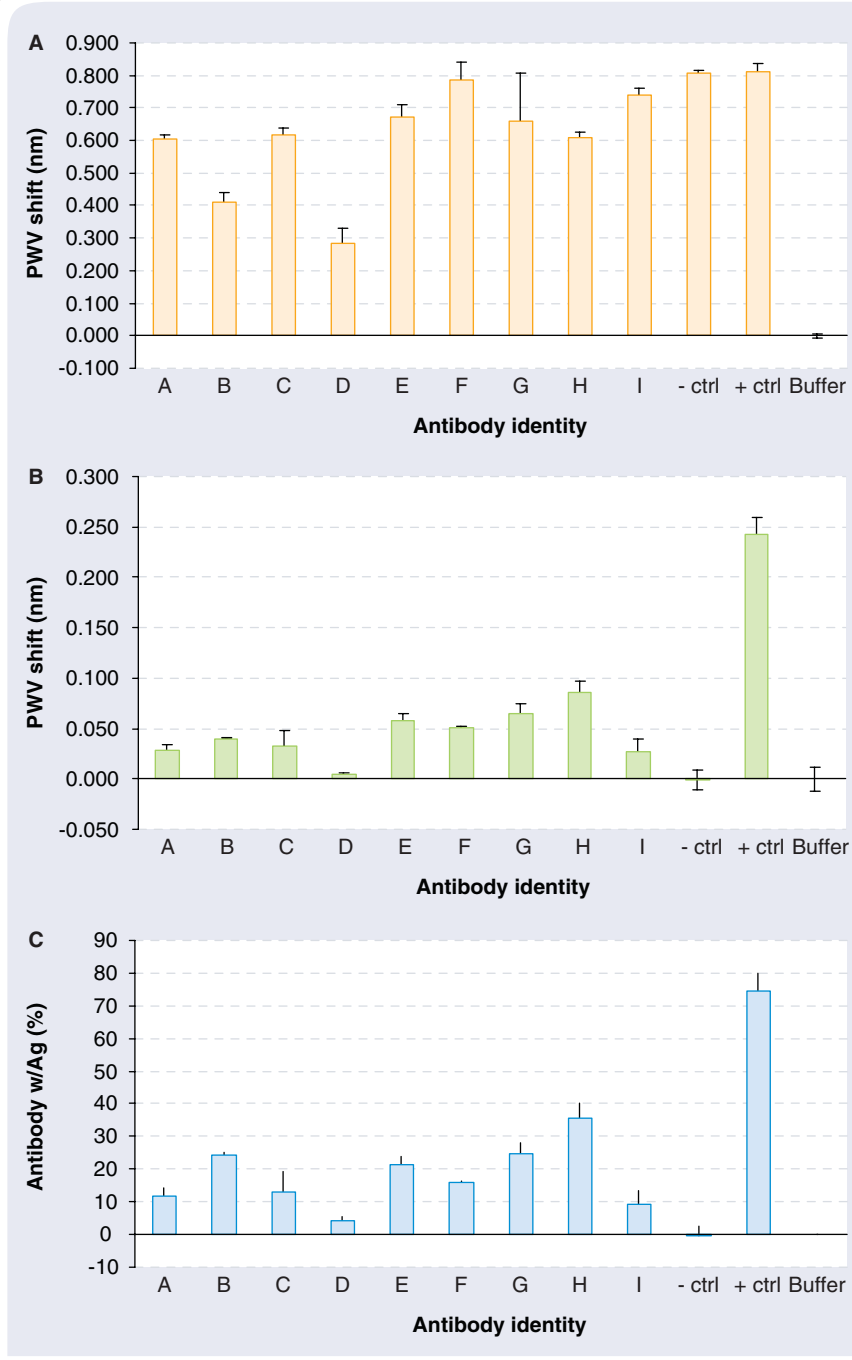

Figure 5. (A) Capture and quantification of the antibody. Nine antibodies (Mr ~150,000) are captured in different wells on the BIND™ sensor along with a strong positive control and a nonspecific negative control. The PWV immobilization level is directly related to the amount of each antibody that is captured. (B) Capture and quantification of the antigen. Antigen (Mr >10,000) was added to the nine antibodies that were captured in different wells in the previous step. The PWV immobilization level is directly related to the amount of antigen that is captured in each well. The controls are performing exactly as expected and each of the nine antibodies is capturing different amounts of antigen. (C) Determination of affinity is made by calculating the percentage of one antibody population in a well that is occupied by bound antigen. This is accomplished in a straightforward manner because the PWV shifts from each of the two previous steps correlate directly with the amount of each protein bound to the sensor at each addition step. Using the molecular weight of each species, the calculation of the percentage of bound protein can be made.
- ctrl: Negative control; + ctrl: Positive control; PWV: Peak wavelength value; w/Ag: .

Table 1. Overview of the SRU Biosystems, Inc.'s BIND™ label-free epitope-binning process.

Step	Process	Outcome
1	Capture first antibody	Concentration
2	Add antigen	Affinity
3	Remove unbound antigen	Off-rate/stability
4	Capture second antibody	Affinity/epitope map
5	Remove unbound second antibody	Off-rate/stability

Note: The label-free BIND™ biosensor enables quantitative analysis of binding as each addition is made to the epitope-binning reaction. This is in sharp contrast to an enzyme-linked immunosorbent assay-like approach where the cause of a null result at Step 4 is not easily explained (i.e., could be the result of the antigen not being present or the second antibody is prevented from binding).

than 9 µm resolution. The imaging mode can be used for applications that increase the overall resolution and throughput of the system, such as label-free microarrays, imaging plate reading, self-referencing microplates and multiplexed spots per well [18].

Photonic crystal biosensors & instruments

One of the first implementations of a photonic crystal biosensor has been recently demonstrated using 1D and 2D periodic structured surfaces produced on glass substrates and on continuous sheets of plastic film. The crystal-reflected peak wavelength value (PWV) is determined with 0.5 pm resolution by illuminating with white light at normal incidence, and gathering reflected spectra with a low-cost spectrometer. Previously published work demonstrate that the resulting mass detection sensitivity of less than 1 pg/mm^2 (obtained without 3D hydrogel surface chemistry) has not been demonstrated by any other commercially available biosensor [19,20].

A fundamental advantage of the photonic crystal biosensor is the capability for inexpensive mass manufacturing from plastic materials in continuous processes at 1-2 feet/min. As illustrated in FIGURE 1, the periodic surface structure is fabricated from a low-refractive-index material that is over-coated with a thin film of higher refractive-index material. The surface structure is replicated within a layer of cured epoxy from a silicon-wafer master mold (i.e., a negative of the desired replicated structure) using a continuous-film process on a polyester substrate. The manufacturing process results in a continuous plastic sheet of photonic crystal biosensors over 1000 m long, with TiO_2 dielectric as the active surface material [17]. Microplate sections of 3 × 5 inches are cut from the sensor sheet, and attached to the bottoms of bottomless microplates with epoxy. Using this approach, photonic crystal sensors are mass-produced on a square-yardage basis at very low cost.

Optical fiber-based detection instrument

The first-generation detection instrument for the photonic crystal biosensor is simple, inexpensive, low power and robust. A schematic diagram of the system is illustrated in FIGURE 2A. In order to detect the reflected resonance, a white light source illuminates an approximately 1-mm diameter region of the sensor surface through an optical fiber at normal incidence through the bottom of the microplate. A detection fiber is bundled with the illumination fiber for gathering reflected light for analysis with a spectrometer. Eight illumination/detection heads operate in parallel so that all 96 wells in a microplate can be measured in approximately 15 s. This is limited by the rate of the motion stage.

Table 2. Data from a BIND™ label-free 8 × 8 epitope-binning experiment.

Average shift (pm)		Antibody ID	BIND™ signal values							
Antibody 1	*Antigen*		*Antibody 2 (pm)*							
			A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
238.71	97.17	*A*	0.00	19.49	101.16*	5.28	114.70*	7.31	100.27*	8.19
245.04	6.97	*B*	1.45	0.00	-8.96	-9.42	-1.14	-8.03	-16.28	-6.88
208.85	49.07	*C*	65.16*	16.34	0.00	21.36	60.53*	40.97‡	55.55*	59.30*
213.51	-0.14	*D*	8.32	11.92	1.15	0.00	11.06	-3.72	-0.26	3.80
219.86	90.63	*E*	148.46*	79.18‡	121.68*	65.69‡	0.00	100.59‡	123.57*	131.66*
215.40	1.86	*F*	16.63	14.67	2.92	0.54	13.98	0.00	-0.04	2.74
206.75	59.03	*G*	90.40*	17.70	84.17*	30.39‡	93.18*	54.54‡	0.00	88.48*
233.56	53.83	*H*	18.76	30.57‡	74.56*	7.85	88.46*	15.86	62.28*	0.00

Note: The BIND™ signal values are for the immobilization of an antibody (M_r ~150,000) on the sensor (antibody Layer 1) and the subsequent capture of the antigen (M_r >10,000) on that antibody for each row of a microtiter plate. The values in the table are the BIND signal for the addition of antibody Layer 2. Typically the antibodies and antigens are used at 1–10 µg/ml concentrations, with or without hybridoma media.
*Antibody binds antigen in both orientations.
‡Antibody only binds antigen when antibody is in solution.

Imaging detection instrument

The single point illumination/single point spectrometer detection method described above can be extended to incorporate an imaging spectrometer that is capable of generating high-resolution spatial maps of the PWV on the photonic crystal surface. This capability is possible due to the high degree of lateral optical confinement for photons resonantly coupled into the structure. Using this instrument, it is possible to observe patterns of biomolecule receptor attachment and hybridization interactions with high density. Since white light illumination is used, and because there is no optical contact required (such as a coupling prism) to the sensor, the imaging method can be performed on large sensor areas, such as entire microplates and microarray slides. As the same biosensor structure and peak-detecting method are used for single-point-based and imaging-based detection, the sensitivity (in terms of amount of PWV shift observed and resolution of PWV shift detection) of the approach is not compromised.

A schematic diagram of the biosensor PWV imaging instrument is illustated in FIGURE 2B. To generate a 2D image of the sensor, a motorized stage translates the sensor in the direction that is perpendicular to the image line. The spatial separation of the image lines is determined by the step-size of the stage between each image-line acquisition. Using this technique, a series of lines are assembled into an image. A large area can be scanned in a tiled fashion by translating the sensor in steps along the image-line direction.

Typically, a biosensor experiment involves measuring shifts in PWV, so the sensor surface is scanned twice, once before and once after biomolecular binding, and the images are aligned and subtracted to determine the difference in PWV as detected by the sensor. This scanning method does not require the PWV of the imaged surface to be completely uniform, either across the surface or within a set of probe locations, or tuning of the sensor angle to a resonance condition as with surface plasmon resonance imaging [21]. FIGURE 3 illustrates label-free biosensor images of PWV shift taken by pixel-by-pixel subtraction of a baseline PWV image from a PWV image captured after immobilization of protein (FIGURE 3A) or cells (FIGURES 3B & C).

Applications

Proteomics has been described in several recent reviews as the investigation of all proteins, their various modifications, and their activities in a system and, to some larger and more economically important extent, changes to these systems when members are targeted for therapeutic effect. Current *in vitro* proteomics methods that use labels suffer from at least two fundamental issues:

- Failure to meet the requirement for quantitative measurement of protein concentration and specific activity of all materials used in an experiment
- Inability to make measurements with complex systems, such as cells or extracts in a highly parallel manner

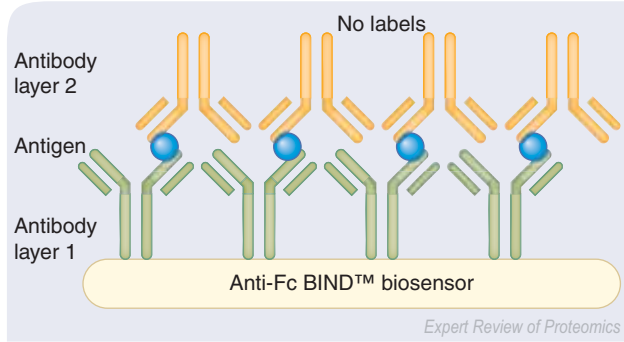

Figure 6. Each antibody is tested for binding to antigen in both orientations: on the sensor as Layer 1 and in solution as Layer 2. This competitive-style binding reaction assesses epitope binding-site similarity, overlap or other types of hindrance to simultaneous binding by both antibodies. A null result for antibody Layer 2 binding could be caused by lack of antigen binding or fast antigen off-rates. The BIND™ sensor enables the build-up of complex interacting protein structures, a process that is critical to proteomics, to be monitored.

Using data examples that address these issues (e.g., hybridoma screening, epitope binning and mapping, small-molecule screening and cell-based functional assays), the label-free BIND system will demonstrate robust abilities to address these fundamental proteomics issues. All assays were performed with positive and negative controls for assessing specific activity of each attached or binding component, and may also have included specific solutions to reduce signals to specific binding components.

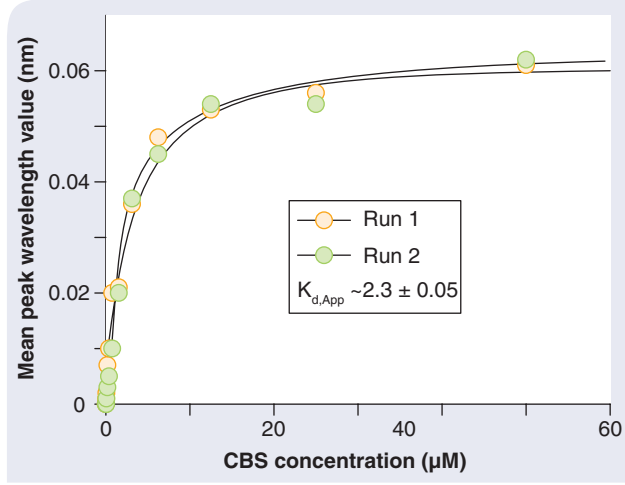

Figure 7. Small-molecule direct measurement of binding to immobilized protein. Carbonic anhydrase II (Mr 29 kDa) is immobilized onto the BIND™ sensor, and different concentrations of a sulfonamide compound (CBS: Mr 201 Da) are added to several columns of wells to perform a binding titration. The data shown here represent two different titrations performed on different days, which demonstrate high reproducibility. An apparent binding affinity is reported here from a single exponential fitted curve that is in good agreement with values in the literature measured with surface plasmon resonance systems.
$K_{d,App}$: .

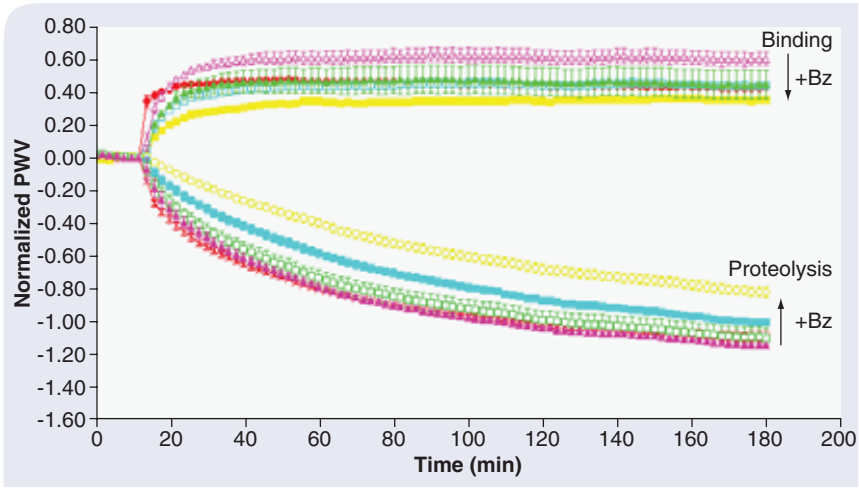

Figure 8. Small-molecule indirect measurement of binding to immobilized protein. Reaction time courses are illustrated for the experiment where soybean trypsin inhibitor (Mr 20 kDa) or a trypsin proteolysis target is immobilized on the BIND™ sensor surface, and trypsin (Mr 24 kDa) is added with increasing amounts of its small-molecule ligand Bz. The titration of the small-molecule ligand for trypsin has a dose-dependent effect on the ability of trypsin to either bind the soybean trypsin inhibitor protein (increasing PWV shifts as additional mass attaches to the sensor), or have effective proteolysis of the target on the sensor (decreasing PWV shifts as mass is removed from the sensor surface). The increasing amounts of the small-molecule ligand are indicated by the direction of the arrows.
Bz: Benzamide; PWV: Peak wavelength value.

Hybridoma screening

Current primary antibody hybridoma screening is performed using enzyme-linked immunosorbent assay (ELISA)-based (label) methodologies and shares similar lack of quantification issues found in proteomics experiments. Antigen is immobilized on a microplate surface and antibody is captured on the surface. The antibody presence is verified using a second antibody that typically recognizes the constant region of the first antibody and signals this binding interaction via a label on the second antibody (typically an enzyme that produces light following addition of a chemical substrate). The amount of the antigen, the first antibody on the plate surface and their specific activities are all unknown. Therefore, the amount of signal the second antibody produces cannot be quantitatively related to the affinity of the first antibody for native target, but is relative to the concentration of the first antibody. FIGURE 4 describes the problem that can lead to prioritization of an antibody whose only quality is high concentration in the hybridoma media. Other formats on this ELISA theme suffer similar shortcomings. Some researchers have suggested purifying the antibodies from the hybridoma media and setting their concentrations to be approximately equal. This is a laborious and time-consuming procedure where reagent is already limiting, and still does not address what becomes of antibodies at low concentration. A process that is supposed to be finding high-affinity proteins is in fact tuned to concentration if these label-assay formats are used. The BIND biosensor can be used to quantify each material that is bound to the sensor at each step. A BIND assay can capture and measure the antibody from complex media. In the same manner, capture and measurement of the antigen interaction can be made in one more step. A simple ratio of the normalized amounts of each of these two proteins can be used to rank the affinity of the antibody (FIGURE 5). In addition to greatly enhancing the knowledge for prioritization of hits in the hybridoma screening process, this type of assay would be immensely useful in proteomics studies where the presence and concentration of a protein on a solid surface is not known and can vary widely from one day, preparation and laboratory to another.

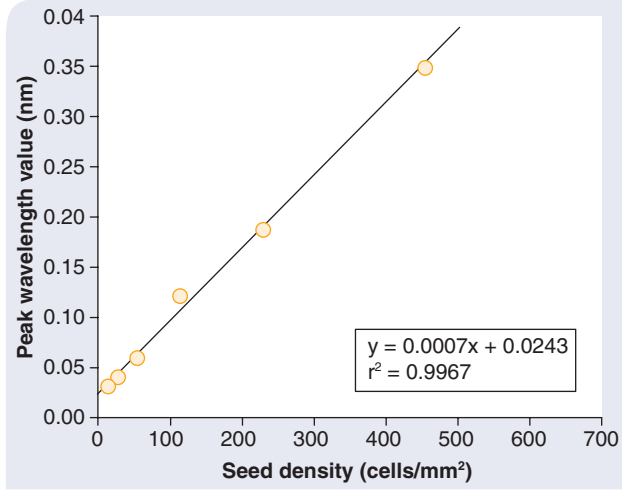

Figure 9. Peak wavelength value (PWV) shift linearity for cell quantification. The data in the graph above demonstrate the high linear correlation between the PWV shift and the seed density for Chinese hamster ovary cells between a few hundred to tens of thousands of cells per well. This correlation has also been extended to include small to large morphological changes of the cells.
r^2: Correlation coefficient.

Epitope binning & mapping

An important part of proteomics as well as therapeutic antibody development is the determination of where and how other molecules a particular protein or set of proteins is interacting. Furthermore, at a more refined level of proteomics study, this determination would provide the detail of exactly what part of a protein is involved in interactions or processes with other proteins or molecules, since as many proteins are involved in multiple interactions with other proteins. Both the interaction with other molecules and the location of those interactions can be readily determined using the BIND system. In a competition-style binning experiment, each antibody is tested against another member of the set for inhibition of binding to

an antigen protein (TABLES 1 & 2, FIGURE 6). This process is designed to determine near or overlapping binding sites on the antigen. A proteomics study could be set up in a similar fashion to determine complex relationships between interacting proteins without the need for separate labels for each test-set member. Additional detail regarding exact binding site amino acid location can be gained by employing truncated protein fractions or series of synthetic peptides with single amino acid differences.

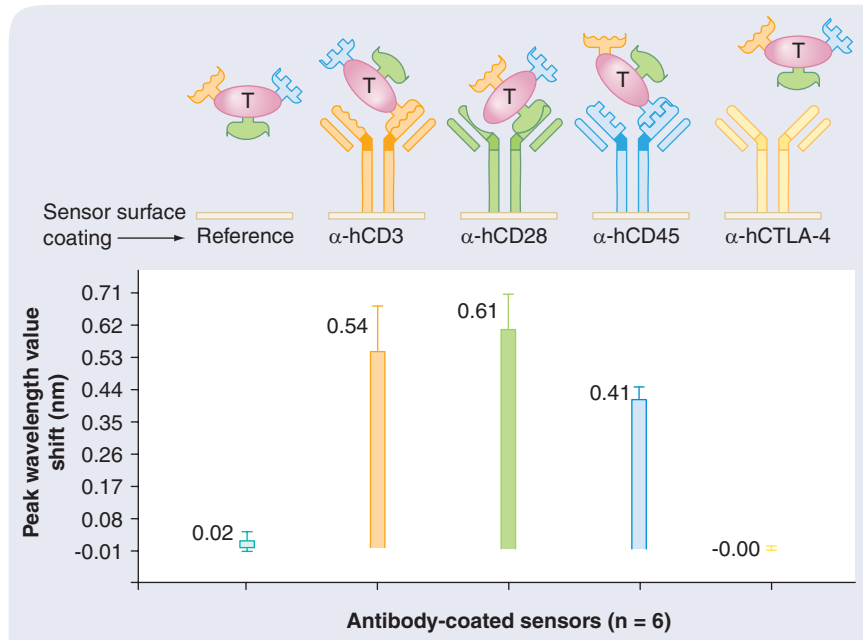

Figure 10. Nonadherent cells can be identified and quantified for specific native cell surface protein production. The end point data from a BIND™ label-free experiment demonstrate the capability for the system to identify, capture and quantify cells expressing specific proteins on their surface. For the experiment, the sensor was coated with an antibody (Mr ~150,000) that recognizes the protein present on the cell surface. Unbound antibody was removed from the well and, subsequently, approximately 10,000–100,000 Jurkat cells were added to the antibody-coated wells. Where no antibody was coated or antibody for a nonexistent cell surface protein was coated, no signal was obtained when the Jurkat cells were added to the well. The same type of experiment can also be performed with adherent cells.

Small-molecule screening

An important part of proteomics has become the understanding the complex interactions of small molecules with proteins (often called chemigenomics or chemiproteomics) for therapeutic purposes. To date, there have been no small-molecule plate-based screening assay formats that are capable of directly measuring the binding of molecules as small as a few hundred Daltons to a protein target, and none can measure massive, parallel protein targets. Most attempts to label the small molecule significantly change the fundamental chemical characteristics, due to the small size of the molecule, or present extreme difficulty of individually labeling millions of compounds. Current small-molecule screening formats indirectly measure the binding of small molecules by looking for agonism or antagonism of interaction with some substrate or binding partner, or change in some other biophysical property of the target. Data in FIGURE 7 demonstrate that BIND can measure small-molecule binding directly to an immobilized enzyme in a microplate. This has been demonstrated with enzyme and nonenzyme target proteins on protein-spotted surfaces as small as 100 µm in diameter, suggesting that large-scale parallel formats are accessible. In addition, the BIND system is even more capable of measuring indirect binding of the small molecule in complex media (FIGURE 8), thus providing the necessary bridge between single target screening and screening of a target in a more complex system, which one would expect to find in therapeutic development processes.

Cell-based assay

The label-free biosensor system described here is versatile, since cell study for proteomics is accommodated on the same plate-based sensor and instrument that protein–protein and protein–small-molecule interactions are performed. Due to expression difficulties, many proteins cannot be studied outside their native environment on a membrane surface. High-level

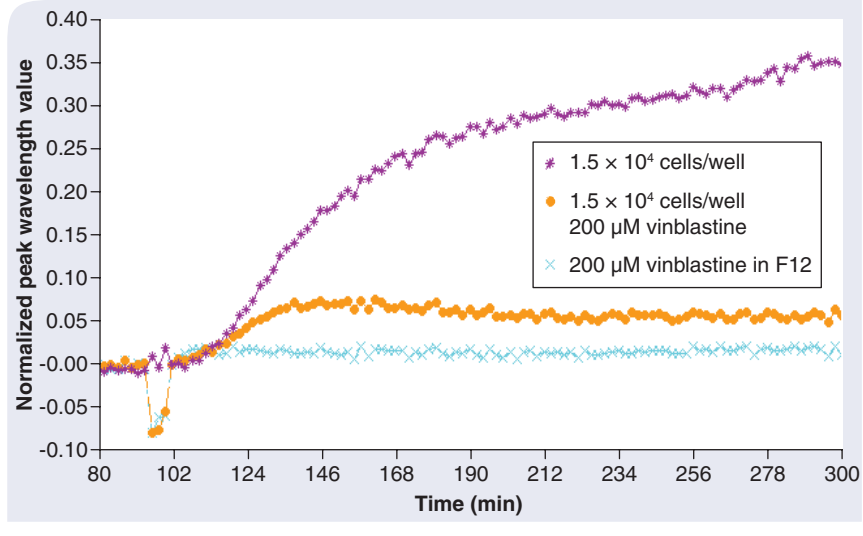

Figure 11. Measuring the effect of the interaction of a small molecule with cellular target. Vinblastine was mixed with Chinese hamster ovary cells in F12 media prior to seeding into wells on the BIND™ sensor. Data are shown for the time course following the addition of the cells to the sensor wells. The effect of vinblastine limits the cells attachment to the sensor.

proteomics functional data in complex environments can only be gathered in the context of the whole cell system. Surface protein expression and interaction with other cellular components are demonstrated in the BIND data below. The data points in FIGURE 9 illustrate the high linear correlation between the BIND signal and the number of cells seeded onto a sensor. In addition, identification and quantification of cells expressing specific proteins on their surface is seen in FIGURE 10. Small molecules or proteins that effect cell surface protein expression or lead to morphological changes can be measured with the BIND label-free system, as demonstrated in FIGURE 11.

Expert commentary & five-year view

As the short list of examples demonstrates, label-free detection may be applied widely in the drug-discovery pipeline from basic research of protein–protein interactions, through primary screening, secondary screening, functional assays, clinical trials and manufacturing quality control. The BIND label-free system is the single platform capable of quantitative analysis of the entire process from target identification and characterization through drug discovery and development. The main technical hurdles to widespread adoption of label-free detection have been lack of sensitivity, quantitative analysis and throughput, high cost per assay, and instrument complexity. The first generation of products based upon photonic crystal label-free optical biosensors already demonstrate the ability to detect low-molecular-weight chemical compounds binding to high-molecular-weight proteins and the ability to image and detect attachment, proliferation and apoptosis individual cells in the same sensor and instrument platform. Similar to the way that silicon transistors have evolved from their early embodiments to today's high-performance integrated circuits, photonic crystal biosensors will also continue to develop as new design features, materials and instrumentation approaches further push the limits of sensitivity and detection resolution. Devices with five-times higher sensitivity performance than the sensors used in the assays reported in this review have already been routinely demonstrated in the laboratory [22], and hand-held instruments with higher resolution than the spectrometer-based approaches reported here are under development. These advances, combined with advances in high-density surface chemistry, microfluidic integration and imaging instruments that can automatically reject common sources of error (e.g., nonspecific binding and bulk refractive index changes) are leading to the capability to resolve the binding of small numbers of protein molecules to the sensor surface.

Over the next 5 years, it is expected that the technology will be adopted for an increasingly wide range of applications, some of which will be new assays that could not be performed efficiently by other label or label-free methods, while some applications will simply replace methods that are currently routinely performed by a labeled assay that is more time consuming and

Key issues

- Label-free optical biosensors in a microplate format enables assays for:
  - Protein screening:
    - Concentration, affinity and activity
  - Small-molecule screening:
    - Assay development, primary, secondary and later development
  - Cell screening:
    - Proliferation, cytotoxicity, size change and movement
- BIND™ is a common platform with high throughput, low cost per assay and high sensitivity.
- A photonic crystal subwavelength optical biosensor structure results in strong interaction between immobilized biomaterial and a highly confined electromagnetic field. The device structure is inexpensively produced from continuous sheets of plastic film.
- Since light is confined from lateral propagation in the photonic crystal, an imaging instrument can measure the spatial distribution of detected material with less than 9-µm pixel resolution across the entire microplate surface.
- The imaging detection capability enables self-referencing against nonspecific assay errors (including bulk refractive index variability), multiplexed assay spots within a single well, microarrays and label-free cell imaging.
- Protein–protein interaction applications include hybridoma screening and epitope mapping.
- Sensitivity and resolution is sufficient for label-free detection of small molecule analytes (Mr<200 Da) by immobilized proteins and enzymes. Characterization of K_d is performed rapidly using several microplate wells in parallel.
- Cell study for proteomics is accommodated on the same microplate-based sensor and instrument that protein–protein and protein–small-molecule interactions are performed.
- Characterization of the interaction between proteins expressed on the cell surface with immobilized proteins on the sensor surface is an increasingly important application of the technology.

complicated. The ability to monitor specific interactions in complex media without labels will greatly expedite the process of detailing the human proteome. Combinations of this label-free technology with other instrumental techniques, such as mass and fluorescence spectroscopy, have already been contemplated and tested. Advances in these areas will provide truly universal detection, quantification, and identification methods for any type of molecule in any type of media.

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- •• of considerable interest

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