Spectral Reflectance Imaging for a Multiplexed, High-Throughput, Label-Free, and Dynamic Biosensing Platform

Emre Özkumur, Student Member, IEEE, Carlos A. Lopez, Ayça Yağcın, Student Member, IEEE, John H. Connor, Marcella Chiari, and M. Selim Ünlü, Fellow, IEEE

(Invited Paper)

Abstract—There are a number of emerging optical biosensing techniques utilizing interferometric and resonant characteristics of light. We have recently demonstrated an interferometric technique, the spectral reflectance imaging biosensor (SRIB) that uses optical wave interference to detect changes in the optical path length as a result of capture of biological material on the microarray surface without the need for labels and secondary reagents. In this paper, we review the principles and performance of the SRIB technique in the context of label-free biosensors and demonstrate its high-throughput, quantitative and calibrated, versatile, and dynamic (kinetic) capabilities. A unique aspect of the SRIB system is that the measurement technique is independent of surface conformation and allows for utilization of novel polymeric coatings for surface binding, thus providing a versatile and high-density platform. We present experimental results on multiplexed antibody/antigen arrays and DNA hybridization in real time, as well as specific binding of whole virus particles. The simplicity of the overall system, its high sensitivity and compatibility with glass surface chemistries, and a linear dynamic range of nearly four orders of magnitude makes SRIB a promising platform for multiplexed detection of different biological analytes in a complex sample, with potential impact in research and clinical applications.

Index Terms—Biosensing, high-throughput, interferometry, label-free, microarrays, quantitative detection.

I. INTRODUCTION

LIVING organisms function as a result of a vast network of macromolecular interactions, including those between antigen–antibody, receptor–ligand, virus–cell, and protein–DNA. Proper cellular function and survival is largely the collective effect of continuous reversible binding events between these biological entities. Therefore, knowing the binding affinities between biomolecules and detecting the presence, absence, or amount of biomolecules are essential for understanding and controlling both cell physiology and disease progression. For this reason, research in medical and life sciences benefits greatly from improvements in the biosensing technologies.

Spectral reflectance imaging biosensor (SRIB) was introduced recently as a promising technique for performing label-free, dynamic, and high-throughput biosensing [1], [2]. Surface mass sensitivity of $\sim 5$ pg/mm$^2$ and concentration sensitivity of approximately nanograms per milliliter of antibody in buffer was demonstrated before. So far, only binding of the antibodies to the spotted antigens was demonstrated with this system. In this paper, after a background on high-throughput and label-free sensing, a detailed description of the technique is given. Then, to demonstrate the potential of utilizing SRIB for a variety of applications, proof-of-principle experiments for detecting antibodies, antigens, oligonucleotides, and whole viruses are shown.

A. Motivation for High-Throughput Detection

Experiments in the fields of medicine, biology, and drug discovery drive a demand for improved biosensors. As the investigated networks are extremely complex and involve a vast number of interactions, scientists are interested in simple and high-throughput analytical methods, such as microarrays. Microarrays were first introduced for RNA expression level studies in the mid 1990s. The small volumes needed to immobilize the probe single-stranded DNA (ssDNA) and small amounts of mRNA extract needed for slide incubation, as well as the high density of the arrays were a major advancement in the speed of expression profiling [3]. Introduction of in situ synthesis of DNA strands on glass surfaces for DNA microarray fabrication has enabled analysis of millions of spots on a single microscope slide [4].

The significance of high-throughput platforms has been demonstrated by the success of gene arrays in the analysis of nucleic acids for many important applications, including medical diagnostics and cancer research [5]–[9]. This success has further provided impetus for extending the use of microarray platforms to perform interaction analysis of DNA and protein transcription factors (TFs) [10], [11], as well as proteins with other proteins [12], [13]. Protein microarrays have received great interest in the last decade.
interest from the biological and medical research communities [14]–[18] and their applications have expanded to potential use in diagnostics [19]–[21] and biohazard detection [22].

Despite this excitement, protein microarrays face many challenges that are not encountered by DNA microarrays [23]. First, proteins are more complex and demonstrate a higher degree of variability than their nucleic acid counterparts, and thus, cannot be synthesized as easily. Furthermore, handling and storing proteins always require more attention. Therefore, a significant research effort across various disciplines is addressing these challenges. Two examples are the “nucleic-acid-programmable protein array” (NAPPA) and “DNA array to protein array” (DAPA) techniques, which have recently been introduced [24], [25]. These two techniques rely on a similar idea in which proteins are synthesized in situ from coding double-stranded DNA and captured on the surface in the vicinity of DNA spots to form the protein array.

Biomolecular interactions greatly depend on the conformation of the interacting molecules and this poses another challenge for protein microarrays. Immobilization on solid surfaces may cause them to denature, thus affecting their affinity for other proteins. DNA arrays that retain optimal protein binding activity are also desirable for more sensitive detection of TF interactions. Better and simpler surface functionalization techniques under investigation allow higher densities and more functional immobilization of biomolecules on the surface [26]–[28]. Toward this goal, Chiari et al. have introduced a novel polymeric coating that increases the immobilization density and functionality of the spotted proteins and nucleic acid probes [29], [30]. Yalçın et al. demonstrated that the volume of this polymeric coating increases by swelling in aqueous environments, thus possessing a 3-D structure that mimics a solution-like state [31].

While a significant amount of research is performed to improve the microarray deposition methods, there are efforts to improve the detection systems as well. Label-free imaging methods are under investigation for more reliable and accurate detection of microarray experiments.

B. Motivation for Label-Free Detection

Traditional microarray imaging commonly employs tags (mainly, fluorescent molecules) that are attached to the captured analytes to create a detectable signal. The binding of target molecules to the immobilized probes are visualized by imaging the surface with a fluorescence scanner. Despite its popularity, it is generally acknowledged that this method has inherent drawbacks, and that for many, if not for most applications, label-free detection would be highly desirable. Even though fluorescence detection generally performs with superior sensitivity, there are applications for which label-free detection sensitivity is sufficient and label-free detection is required [16], [23], [32]–[34].

One of the drawbacks of labeled detection is that labels might modify the interaction affinities of the macromolecules to which they are attached. In some cases, special attention is paid to attach the label to a designated epitope on the protein in order to minimize the effect on binding kinetics. But, frequently, proteins are labeled nonspecifically, and the fluorescent labels may interfere with the binding sites. This is a significant problem when studying small molecules and peptides, as the amino acids involved in binding are more likely to be affected. The effect is clear for larger molecules as well. A recent study by Sun et al. [35] shows how the properties of fairly simple and widely used proteins are affected by labeling. In this study, streptavidin–peptide and antibody–antigen reactions were monitored with a label-free technique, and it was observed that when the target is labeled with Cy3 molecules, the detected reaction kinetics change significantly.

To avoid complications that result from directly labeling the target molecules, sandwich assays are often employed [16], [36]. After the first incubation with target molecules that are not labeled, the array is incubated with labeled secondary probe molecules that are also specific to the target. However, the second incubation should not interfere with the first one, namely, two different probes that are specific for two distinct nonoverlapping epitopes on the same target protein are required for the sandwich assay to work properly [37]. Otherwise, a competition reaction occurs that can create false-negative signals. Also, the secondary probe should not have any specificity for the spotted probes. If this is not satisfied, a false positive may occur when the secondary probes bind to spotted molecules without the initially captured target. In summary, the detection of analytes through secondary probes is intrinsically complex, as it requires multiple layers of interacting components that provide specificity without interfering with one another.

Label-free measurements can provide kinetic information about the reactions, which is not possible with fluorescent techniques. Even though quantification of captured biological mass is possible in fluorescence measurements using on-chip calibration procedures [38], the bleaching of fluorescent molecules avoid real-time data acquisition to characterize the biomolecular interaction thermodynamics. Since the photoluminescence ability of dyes decay over time through excitation, a phenomenon called photobleaching occurs and prevents a linear response during long exposure times.

Labeled detection methods are widely used because of their sensitivity and the historical lack of viable alternatives. However, depending on the application, the challenges they present can be significant and difficult to overcome when a quantitative measure of binding is required. In such cases, label-free detection has major advantages over the labeled detection methods. Besides the advantages listed in this section, label-free methods eliminate at least one chemical step from the assay process. Thus, they are time- and cost-efficient, and reduce experimental variability due to user error.

C. Current and Emerging Biosensing Platforms

Surface plasmon resonance (SPR) has been the leading technology among label-free detection methods. Traditional SPR has been limited to a macroformat with very limited throughput (four or eight channels). However, recent advancements in the technology show that higher throughput is, indeed, possible. Imaging SPR, presented by Shumaker–Parry and Campbell, is capable of imaging interactions for 120 features, in real time,
with 3-pm height sensitivity [39], [40]. In contrast to regular SPR in imaging SPR, both the angle and the wavelength are fixed to the high linear region of system response. The reflection is imaged to a charge-coupled device (CCD) camera, and molecular accumulation on the surface is detected as an intensity change on the camera [41]. This technique is currently going through modifications and adjustments to improve the sensitivity and throughput further [42]. Campbell and Kim, in their review article, discuss a commercially available system that is capable of imaging >1300 spots with ∼3-pm noise floor for each spot, with a time resolution of 1 s. [43]. Although SPR has been quite successful, some of its shortcomings may be prohibitively difficult to overcome, depending on the application of interest. First, imaging SPR has a limited linear dynamic range. As a result, similar initial mass densities and surface uniformity have to be maintained for all the immobilized probes [44]. For the same reason, multiple layers of interactions are hard to monitor with the same sensitivity. Improvements are under investigation to increase the dynamic range of SPR microscopy [45]. Also, the requirement of gold surfaces increases the cost of substrates. Furthermore, a change in the optical properties of the buffer affects the signal (known as “bulk” or “matrix” effect); therefore, buffer-dependent association/dissociation experiments may be problematic to carry out. The system temperature should also be well regulated during measurements, which requires well-designed temperature-control systems to be used for long experiments, thus the instrumentation is further complicated.

As an alternative to SPR, methods that employ interferometric, ellipsometric, and resonant characteristics of light have become popular for biosensing, as they offer simple and sensitive detection. Using molecular interferometric imaging (MII) [46], [47] real-time detection on a 40-spot array was shown with a noise floor of 15–20 pm per spot. Another technique, the biomolecular interaction detector (BIND), has been commercialized by SRU Biosystems. Using BIND, very high sensitivities were achieved and small molecules (<300 Da) were detected in real time [48]–[50]. Dynamic microarray imaging has achieved limited success, but the technology was successfully applied to industry standard 96-, 384-, and 1536-well microplates [51], [52]. Reflectometric interference spectroscopy (RIFS) was first introduced almost two decades ago, and it has been in use since then. A noise floor of a few picometers and small analyte (biotin) detection at a concentration of 40 nM was demonstrated, which compares well to single-channel Biacore SPR systems [53]–[55]. High-throughput detection was also suggested in which a 96-well plate was imaged label free with a noise floor of 20 pm [56]. The oblique-incident reflectivity difference (OI-RD) method is an optical ellipsometry method. This inherently single-point detection method was used for high-throughput detection by scanning either the substrate or the optical illumination and collection system [57], [58]. The resonant cavity imaging biosensor (RCIB) utilizes a Fabry–Pérot cavity formed by two highly reflecting mirrors and it has shown potential for high-throughput detection [59].

Some of the other optical label-free detection techniques that attracted significant interest are porous silicon-based reflectometry [60], resonance sensing [61]–[63], and backscattering interferometry [64], [65]. Important examples of nonoptical biosensor technologies include mass spectrometry, quartz crystal microbalance, microcantilevers, nanowires, and electrical detection methods. A comprehensive discussion of these techniques and their commercial availability are reviewed in [33], [34], [66], and [67].

The research to achieve high-throughput biosensors and the work that is conducted to improve label-free sensing platforms suggest that there are applications that would benefit from alternative techniques. In this paper, we discuss an interferometric technique SRIB, developed by Özkmur et al., in detail [1]. The presented method may be advantageous with its simple and robust structure; it compares well to current state-of-the-art technologies in terms of sensitivity and throughput and performs better in terms of dynamic range.

II. PRINCIPLES OF THE SRIB

A. Basics of SRIB

Utilizing the interference of light that is reflecting from different interfaces of layered substrates has been in use for biosensing for several decades [47], [53], [68], [69]. The basic working principle of these platforms is the use of spectroscopy to quantify the interference signature of light reflected from a layered structure and accurately measure the optical thickness of a designated transparent film. Adsorption of minute amounts of mass (biological entities) alters the layered structure, which changes the spectral pattern, and thus, the accumulation can be sensed.

Commonly used reflection spectroscopy techniques utilize a broadband light source for illumination and a spectrometer for detection. This method is inherently a single-point detection platform, as the spectrum can be collected from a single point only at any time. It was proposed to scan either the substrate or the illumination and collection optics to achieve high-throughput detection [51], [58], [70]. This limits the compatibility of the method with dynamic measurements as the array size increases; for more spots, the scanning time increases accordingly, eventually reaching a level where the rate of interactions becomes faster than the data acquisition.

To achieve high-throughput data acquisition and perform reflection spectroscopy at the same time, we proposed to use a wavelength-tunable laser for illumination and a CCD (or CMOS) camera for detection (see Fig. 1). The layered substrate consists of thermally grown silicon dioxide ($SiO_2$) on silicon (Si). The magnitude of the substrate reflectance at a specific wavelength depends on the optical path length difference (OPD) between the top surface of the transparent layer and the buried Si–SiO$_2$ interface. The transparent film consists of thermally grown oxide and bilayer accumulated on top of oxide. Binding on the top surface increases the OPD between the reflections and shifts the spectral reflectance signature, also visible as an intensity difference at a specific wavelength (see Fig. 2).

After the data acquisition, the spectrum collected by each camera pixel is processed by curve fitting. The reflection of a
The biomolecules are spotted on the SiO$_2$ on Si reflector. The reflected light is recorded by a CCD camera for the whole FOV. The layered substrate is illuminated at different wavelengths and at each wavelength an intensity image is recorded. This way, each pixel of the camera records a wavelength versus reflection data, which is processed to find the total oxide and biolayer thickness.

In this method, the refractive index of the biolayer is assumed to be $n = n_{SiO_2} = 1.45$, and increase in the film thickness is given by

$$\phi = \frac{2\pi d}{\lambda} n_{ox} \cos \theta,$$

where $d$ is the total transparent film thickness, $n_{ox}$ is the refractive index of SiO$_2$, $\lambda$ is the wavelength of the incident light, and $\theta$ is the angle of incidence. The angle and polarization dependence of the Fresnel reflection coefficients vanish for normally incident light ($\theta = 0^\circ$). In this paper, as the numerical aperture (NA) of the collection optics is quiet small (0.08), the light that is collected is predominantly the normally incident light, so this approximation holds well.

Biolayer thickness is found by subtracting a reference oxide thickness from the total transparent film thickness. The reference thickness is found by averaging the values of all pixels included in an annulus surrounding the spot. The inner value is found by averaging all the pixels included in a circle that contains the spot. In this method, the refractive index of the biolayer is assumed to be $n = n_{SiO_2} = 1.45$, and increase in the film thickness is interpreted as binding. Commonly, an approximation of 1 nm – 1 ng/mm$^2$ is used to convert the detected thickness to layer mass density [54]. Recently, we have shown that this approximation holds well for ssDNA and proteins of different sizes [2], and we have more accurately found the conversion factors to be 1 nm – 0.8 ng/mm$^2$ for ssDNA and 1 nm – 1.2 ng/mm$^2$ for proteins. These conversion factors are used to find mass densities from the detected optical thicknesses.

B. Advantages of SRIB

In Fig. 2(a), (1) is used to fit data acquired by 3 pixels imaging regions of the substrate of three different oxide thicknesses. In Fig. 2(b), the intensity images that are taken at different wavelengths of a substrate with 5-nm deep etched square regions and a final image after data processing are shown. Data acquisition and processing in this way has several advantages. First, the irregularities pointed in Fig. 2(b) do not show up in the processed image, which indicates that imaging artifacts are filtered out from the final data as long as they do not have a spectral response.

Also, the system response does not change with the changing optical thickness on the surface, which extends the linear dynamic range. The wide dynamic range and quantitative detection capabilities of SRIB were recently demonstrated [2]. The SRIB response to varying amounts of immunoglobulin G (IgG) protein on the surface is shown in Fig. 3. To accurately relate the SRIB response to biomolecular mass on the surface, we have immobilized known amounts of proteins on the surface. This was achieved by spotting these proteins in high purity salt-free water and the deposited spot volumes were accurately measured [2]. This way, as the spotting concentrations were also known, the total amount of biomolecular mass remaining on the surface after the water is evaporated could be calculated. Clearly, no washing was applied for this measurement. The spotting concentrations of IgG were: 0.63, 0.125, 0.25, 0.5, 1, and 2 mg/mL. For low surface accumulation, i.e., thicknesses much smaller than the wavelength of light, the system response is well within the linear regime. As the surface accumulation increases and the...
Fig. 3. SRIB response to spotted mass. Six different concentrations of IgG protein that vary over a factor of $\sim 32$ are spotted. The spotted mass for each concentration is precisely determined, as described previously [78]. The average height of 20 replicate spots for each concentration was found, and multiplied by the area of the circle that was used to measure the height. Since the average height is a measure of mass density, a direct comparison to spotted total mass is obtained in this way. The SRIB response is linear for the first four concentrations as shown, and a nonlinear behavior is observed as the layer thickness increases. Considering the 5-pm or 5-pg/mm$^2$ sensitivity, this corresponds to a linear dynamic range of approximately four orders of magnitude.

Layer thickness becomes comparable to the wavelength, a nonlinear response is observed. In Fig. 3, system response is still linear at surface accumulation of 200 pg. Taking the spot radius of 70 $\mu$m into account, this value corresponds to 13 ng/mm$^2$ surface density. Considering the 5-pg/mm$^2$ sensitivity of SRIB reported earlier [2], it is safe to say that the SRIB response is linear within a dynamic range of four orders of magnitude.

Change in the refractive index of the incubation buffer affects the reflection coefficient $r_1$, but does not alter the detected phase $\phi$. Therefore, SRIB response is independent of temperature and refractive index changes in the bulk solution (termed “bulk effect”), thus allowing utilization of the technique for temperature- and buffer-dependent experiments. A possible thickness change in the oxide layer as a result of temperature changes in the environment is accompanied by a reverse change in the refractive index. Thus, temperature changes in the buffer or environment do not affect the detected optical phase, which eliminates the need for complex and bulky temperature stabilization systems.

Another advantage of the proposed method is the utilization of thermally grown oxide on Si. The uniformity and smoothness of these surfaces are markedly better than conventional glass surfaces. Thus, one can achieve repeatable results in surface functionalization, which is critical for the sensitivity of a detection system, as it depends greatly on functional probe density and surface uniformity. Also, the substrate cost is significantly lower than the gold-coated substrates.

C. SRIB Setup

The configuration of the SRIB setup is shown in Fig. 4. Since the sensing modality is based on imaging the optical thickness, smoothness of the layered substrate and the uniformity of oxide layer are crucial. Si samples were chemically and mechanically polished to better than 0.4 nm roughness, as measured by atomic force microscopy (AFM), followed by thermal growth of the oxide layer. Thermal oxide growth is self-limiting and highly uniform, virtually eliminating noise associated with variations in the oxide thickness. A fiber-coupled tunable diode laser (NewFocus TLB-6300) is used for illuminating the sample. A CCD camera (QImaging-RoleraXR) records an intensity image at each wavelength.

An imaging system with zooming capability was used to match the field-of-view (FOV) to varying array sizes. Current configuration can change the magnification from 0.35$\times$ to 2.25$\times$. The pixel size of the camera is 13.7 $\mu$m and 500 $\times$ 500 pixels are used for imaging. When a magnification of approximately 1.15$\times$ is used, the FOV would be an area of 6 mm $\times$ 6 mm.
The laser is coupled to a fiber coupler of which the reference arm is connected to an external photodetector (PD) for measuring the laser power during data acquisition. The intensity measured by the camera is divided by the PD reading for balancing the acquired data. The laser, PD, camera, and image grabber were controlled with National Instruments Labview software.

Although using a laser source has the advantage of improved wavelength sampling accuracy, its highly coherent nature creates artifacts when used for imaging. The spatial coherence of the laser manifests itself as speckle patterns when reflected from a surface. Also, the imperfections in the illumination and imaging systems create diffraction patterns on the image. To overcome this problem, the laser illumination is passed through rotating ground glass disks by which the degree of spatial coherence of the laser is significantly reduced and related artifacts are cleaned from the detected image [see Fig. 4(b)].

Sensitivity of the presented method was measured by performing repeated measurements on the same sample and monitoring the deviation in the measured mass densities. Because the system performs near shot-noise limited detection, averaging of images at every wavelength and pixels for every spot reduces the noise floor. Assigning more pixels to spots reduces the throughput of the detection, whereas averaging more images increases sampling time of the detection. Therefore, there are tradeoffs between sensitivity—throughput and sensitivity—dynamic detection capability. For the current system in dry conditions, the noise floor was measured as 5 pg/mm² when imaging ~1000 spots with a time resolution of ~30 s. Although sensitivity to target concentration determines if the system is suited for any specific application, mass density type of sensitivity characterization is helpful for better comparison of the technique with other label-free detection methods.

III. DETECTION OF BIOMOLECULAR INTERACTIONS WITH SRIB

A. Surface Activation

Surface functionalization plays a crucial role in microarray technologies. The method of immobilization should allow specific attachment of probe molecules to the surface while maintaining functionality. Surface functionalization techniques should avoid or at least reduce nonspecific binding of target and nontarget molecules to both background and probe locations. An ideal surface functionalization method should also provide a high density of probe molecules that improves the target-detection sensitivity. A uniform and repeatable spot morphology is also desired because this can improve the sensitivity and simplify signal and image processing. Finally, an ideal surface preparation technique should be low cost, robust, simple, and repeatable. Label-free imaging poses an additional parameter such that the surface should remain very clean and smooth and repeatable. Label-free imaging poses an additional parameter such that the surface should remain very clean and smooth and repeatable. Label-free imaging poses an additional parameter such that the surface should remain very clean and smooth and repeatable.

In our proof-of-principle experiments, we have used a novel polymeric coating that was developed by Chiari and coworkers [29], [30]. The polymeric coating, copoly(dimethylacrylamide–acyrloyloxy succinimide–3-(trimethoxysilyl)-propylmethacrylate) (DMA–NAS–MAPS) is a ter-polymer obtained by radical copolymerization of dimethylacrylamide with cyrloyloxysuccinimide and 3-(trimethoxysilyl)propyl methacrylate. It self-adsorbs to the SiO₂ surface forming a stable hydrophilic coating. Each monomer has a specific function in the coating: DMA provides self-adsorption to the SiO₂ surface through hydrogen bonding and van der Waals forces, MAPS increases the strength of the binding through covalent attachment to the SiO₂ surface with silane functionalities, and NAS provides functional groups (NHS esters) that covalently bind the amine groups of the probe molecules.

The polymeric coating improves the probe density and functionality by forming a 3-D structure by swelling upon hydration [31]. The surface coating procedure is simple and repeatable. It also avoids nonspecific attachment of biomolecules on the surface. The functional groups hydrolyze when left in humid environment losing their activity; therefore, the biomolecules are not captured by the coating itself after overnight incubation in humid chamber and washing steps. We have seen no detectable background binding during protein and DNA hybridization experiments. The bovine serum albumin (BSA) blocking that was used in antibody and antigen capture experiments was primarily applied to block the flow elements. We did not detect nonspecific binding to control spots for protein and DNA experiments. However, there was a significant amount of nonspecific attachment of whole viruses to control spots. We believe this was caused by the fact that viruses were labeled with fluorescent markers for control purposes, which made them stickier.

The basic procedure to form the polymeric coating is as follows. The SiO₂ on Si substrates are cleaned thoroughly with acetone, methanol, and deionized (DI) water. The cleaned substrates are treated for 30 min with 0.1-M NaOH to prepare hydroxyl groups on the surface for subsequent steps and are then washed thoroughly in DI water. The chips are immersed in the polymer solution (1% w/v in a water solution of ammonium sulfate at a 20% saturation level) for 30 min, washed extensively with DI water, dried with argon, and then baked at 80 °C for 15 min. The polymer-coated chips are kept in a vacuum dessicator until use and are stable for up to three months.

B. Antibody Detection

We have demonstrated that the presence of antibodies in solution can be detected specifically by SRIB. Antigens are spotted on functionalized surfaces and a mass increase on the antigen spots is seen upon incubation with the cognate antibody. Human serum albumin (HSA), rabbit IgG (R IgG), BSA, mouse IgG (M IgG), and hepatitis-B surface antigen (HBsAg) were spotted using a benchtop spotting unit (BioOdysssey Calligrapher miniarrayer, Bio-Rad). HBsAg was purchased from Fitzgerald Industries and spotted at a concentration of 0.25 mg/mL. All other proteins were purchased from Sigma-Aldrich and spotted at a concentration of 1 mg/mL. The spotting buffer was phosphate buffered saline (PBS) (Fisher Scientific, pH 7.4). The wafers were incubated for at least 1 h in the Bio-Rad Calligrapher humidity chamber (55% humidity) to promote antigen
The specific binding of anti-HBsAg to HBsAg, anti-HSA to both HSA and BSA (because of the polyclonal nature of the antibodies and the high degree of homology between HSA and BSA), and anti-mouse to mouse IgG proteins were detected during the initial three incubations. When the sample was incubated with anti-rabbit antibodies, binding to rabbit-IgG was seen, but mass increase on the HSA, BSA, and HBsAg spots was detected as well. Anti-rabbit antibodies that were used in the final incubation bound to initially captured antibodies that were of rabbit origin, thus forming multiple layers of interacting biomolecules. Anti-HBsAg incubation period was 35 min, anti-HSA incubation period was 50 min, anti-Mouse incubation period was 25 min, and anti-rabbit incubation time was 70 min. By introducing the IgG elution buffer, it was shown that measuring the dissociation kinetics is also possible. Finally, all bound molecules, other than the initially spotted/immobilized molecules, were washed off with hydrochloric acid (HCl) incubation (see Fig. 5).

The images that are taken at different time points during the experiment are given in Fig. 5. An initial image is actually subtracted from every data that were taken consecutively. Therefore, spots are not visible, until there is a differential height change corresponding to binding. For the same reason, the dynamic binding curves show differential height change, starting from zero at the beginning of the experiment. The x-axis of the graph demonstrates optical height thickness change with assuming a refractive index of 1.45 for the biolayer. Conversion to mass density is assumed to be $1 \text{ nm} = 1.2 \text{ ng/mm}^2$. It is worth noting that as a result of multilayered binding, the incremental mass immobilization before being stored overnight in 4 °C in the presence of desiccant.

The samples were washed with a standard washing procedure: three times in PBS with 0.1% Tween20 (PBST), three times in PBS, and one time in DI water, for 5 min each. The sample was dried under argon and placed into a custom-built flow chamber that is sealed with a glass window to enable dynamic SRIB detection. After washing the flow elements with PBST and blocking with 1 mg/mL of BSA, the sample was incubated sequentially with rabbit anti-HBsAg, rabbit anti-HSA, goat anti-mouse, and goat anti-rabbit antibodies while the sample was continuously imaged with the SRIB system (see Fig. 5). An initial image is subtracted as a reference image; therefore, the acquired data shows differential mass changes on the spots. All the incubations were done with antibody concentrations of 10 µg/mL in PBST. Between incubations, the sample was washed with PBST. The flow chamber that was used during these experiments was ~2 mL in size and the flow speed was ~ 0.5 mL/min. The incubations took 60 min on average and the solutions were continuously flowed in the chamber. These flow conditions require a reagent volume of 30 mL and 300 µg of antibody for the specified concentration. The amount of reagents used in this experiment primarily depends on the flow conditions and by improvements in the fluidics, it can be reduced significantly.
density goes as high as 15 ng/mm$^2$, but the response of the system and noise characteristics does not change. This is enabled by the wide linear dynamic range of the system.

C. Antigen Detection

For antigen capture, the immobilized probe antibodies should be functional on the surface with their Fab regions exposed to the incubation solution. The polymeric surface coating facilitates an environment, where antibodies have more freedom for movement than the commonly used 1-D surface coatings. Affinity purified antibodies and antigens were spotted on the surface and the presence of specific antigens and antibodies was detected by the same chip during sequential incubations.

Affinity purified anti-mouse and anti-rabbit antibodies were purchased from Jackson Immunoresearch, Inc. Rabbit IgG and anti-rabbit IgG were spotted at a 1-mg/mL concentration, mouse IgG and anti-mouse IgG were spotted with a 0.5-mg/mL concentration in PBS. After an overnight incubation at 4 °C with desiccant, the spots were rehydrated by leaving them in a high humidity chamber for 1 h. After standard washing (3 × PBST, 5 × PBS, and 1 × DI water, each 5 min), the sample was placed in the flow-chamber and the surface and flow elements were blocked by flowing a concentrated BSA solution of 1 mg/mL.

After incubating with 5 μg/mL of mouse IgG in PBST, the mouse antibodies were captured by the anti-mouse IgG antibodies. Following the first incubation, the sample was incubated with 5 μg/mL of rabbit IgG in PBST and rabbit antibodies were captured by the spotted anti-rabbit IgG antibodies. Finally, the sample was incubated with anti-mouse IgG and anti-rabbit IgG antibodies. During this final incubation, binding to the spotted mouse and rabbit proteins, as well as to the antigens captured after the first two incubations was detected (see Fig. 6). The incubation period for antigens was 20 min, whereas the antibody solution was left to incubate for 35 min, as it took more time to saturate.

SRIB images at different time points are given in Fig. 6(b); an initial reference image is subtracted from consecutive images, as it was done in antibody detection experiment. The initial spot mass densities were measured as ~4 ng/mm$^2$ (data not shown) both for the antibody and antigen spots. Upon antigen incubation, the incremental mass change on the specific spots was ~1 ng/mm$^2$ on average. Considering both the probe antibodies and captured antigens have the same molecular weight (150 kDa) and a saturating amount of antigen concentration was used, this result implies that only ~25% of the antibodies were functional on the surface. Even though this ratio is better than what we could achieve by other surface functionalization methods, increasing the amount of functional antibodies would improve the overall sensitivity to antigens. During antibody incubations, the signal is enhanced because polyclonal antibody reagent contains antibodies against different epitopes on the spotted antigens. Therefore, independent of the orientation of the antigen on the surface, there is a population of antibodies in the analyte solution specific to the exposed region, thus increasing the chance of interaction. However, in the case of spotted antibodies, the interaction is possible only when the Fab fragments of the antibodies are exposed.

D. Detection of Whole Viruses

The SRIB system can be used to test for the presence of viruses in solution, which may lead to rapid diagnosis of infectious diseases. By using antibodies specific for viral surface proteins as the probe molecules, whole viruses can be captured on the surface. As the sizes of many viruses are still smaller than the wavelength of light that is used for imaging (~λ/10), scattering from the viruses is predominantly forward scattering and they act as phase objects. Therefore, the optical modeling of reflected light does not change for this application.

To demonstrate proof-of-principle for virus detection, vesicular stromatitis virus (VSV) was used as a surrogate target. VSV does not normally infect humans, and it can be modified to safely express proteins from other viruses on its surface through a process called pseudotyping. It, therefore, presents a simple way to demonstrate detection of more dangerous viruses, such as the highly lethal Marburg and Ebola viruses.

Si wafers were first functionalized with the polymeric coating described earlier, and antibodies specific for the external segment of the VSV surface glycoprotein were then spotted in addition to three other biomolecules used for control purposes [see Fig. 7(a)]. Upon incubation with a VSV solution containing...
Fig. 7. Preliminary results on virus detection. (a) The difference image comparing before and after the incubation with VSV. (b) Results indicate a higher level of binding on the specific spots, although there is a significant level of nonspecific binding to the control spots. This may be attributed to the labeling strategy of the virus, the use of general, non-specific protein probes as controls, and other incubation and spotting parameters.

~10^8 plaque-forming units (PFUs), a clear binding signal is seen on the specific spots, as well as some nonspecific binding to the control spots. This experiment suggests that the mass increase as a result of virus capture can be detected with SRIB and may allow specific detection to be achieved. To prove diagnostic capabilities, further experiments are needed to show that clinically relevant concentrations of the virus can be detected in biological samples and that specific virus capture can be achieved in a multiplexed scenario (mixed virus sample).

E. Detection of Short DNA Strands

Detection of single stranded oligonucleotide chains in solution has various application fields, such as DNA diagnostics for personalized medicine or expression profiling. We have demonstrated that DNA microarrays can be imaged quantitatively and sensitively with SRIB.

ssDNA chains of 10 µM concentration in sodium phosphate buffer (150 mM, 0.01% Triton, pH 8.5) were spotted on the polymer-coated surface. The primary and control ssDNA sequences were 23 nucleotides long, and modified at the 5′ end with an amine group for covalent attachment to the polymeric coating. The spotted specific probe sequence was: 5′-amino modifier C6-GCC CAC CTA TAA GGT AAA AGT GA-3′.

The target oligonucleotide sequence was: 5′-TCA CTT TTA CCT TAT AGG TGG GC-3′. The control sequence was the same with target sequence, but it was amino-modified at the 5′ and to enable immobilization on the surface upon spotting.

The substrate is kept overnight at room temperature in a 75% humidity chamber, and washed twice with 2× saline sodium citrate (SSC)/0.01% sodium dodecyl sulfate (SDS) buffer and twice with 0.1× SSC buffer before measurement. The substrate is then placed in a chamber, and the chamber is initially filled with 2× SSC buffer, followed by 10 nM concentration of target ssDNA in 2× SSC buffer. Fig. 8 shows the label-free, real-time detection of hybridization on the surface at 50 spots, ten of which have control ssDNA present. The percent hybridization is determined with respect to the initial ssDNA immobilized at every spot, and an average hybridization of 55% is achieved at this concentration. Considering the clear signal that was detected upon incubation with 10 nM of target concentration, we believe the limit of detection for short oligonucleotides would be in the subnanometer range.

IV. CONCLUSION

The SRIB has proven to be a powerful tool for label-free sensing for microarray applications. We have demonstrated dynamic imaging of antibody–antigen and DNA–DNA interactions, as well as endpoint detection of antibody-whole virus binding. It is possible to achieve a throughput >10^3 spots with 0.01-ng/mm² mass sensitivity with the current system. The data acquisition takes less than 30 s; therefore, kinetic characterization of interactions is also possible. SRIB presents an alternative to current state-of-the-art assay technologies, with the added advantages of using an inexpensive substrate for probe immobilization, reduced dependence on bulk effects, large linear dynamic range, and quantitative binding determinations.

REFERENCES


clotide microarrays using a digital micromirror array,” Nature Biotech-


Lan, R. Jansen, S. Bidlingmaier, T. Houfek, T. Mitchell, P. Miller, R. A.


J. Dean, S. Fournel, D. Fong, M. C. Genovese, H. E. Neuman de Verguer, K. Skettles, D. D. Hirschberg, R. I. Morris, S. Muller, G. J.
Prujin, W. J. van Vrenouj, J. S. Smolen, P. O. Brown, L. Steinman, and P.


C. Zangar, “Evaluation of surface chemistries for antibody microar-


Marcella Chiari received a B.S. in chemistry and pharmaceutical techniques in 1982 and a Ph.D. degree in clinical biochemistry in 1990, both from the University of Milan, Italy.

She is currently a Senior Research Scientist with the Italian National Research Council, Milan, Italy, where she leads the Analytical Microsystems Laboratory, Institute of Molecular Recognition. She has worldwide recognized experience in development of hydrophilic linear polymers. She is involved in the area of protein and DNA microarray research. Her research interests include the development of innovative polymeric coatings for glass slides. She has been engaged in development of a number of new hydrophilic acrylic monomers and polymers to be used in capillary electrophoresis as DNA sieving matrices and capillary coatings.

M. Selim Ünlü (M’90–SM’95–F’07) received the B.S. degree from Middle East Technical University, Ankara, Turkey, in 1986, and the M.S.E.E. and Ph.D. degrees in electrical engineering from the University of Illinois, Urbana-Champaign, in 1988 and 1992, respectively.

Since 1992, he has been with the Department of Electrical and Computer Engineering, Boston University, Boston, MA, where he is currently a Professor of electrical and computer engineering, biomedical engineering, and physics, an Associate Dean for research and graduate programs in engineering, as well as the Associate Director of Center for Nanoscience and Nanobiotechnology. His research laboratories—Optical Characterization and Nanophotonics (www.bu.edu/OCN)—are located in the Photonics Center. His current research interests include nanophotonics and biophotonics, research and development of photonic materials, semiconductor optoelectronic devices, high-resolution microscopy and spectroscopy of semiconductor and biological materials, and biological sensing and imaging.

Dr. Ünlü was the Chair of IEEE Laser and Electro-Optics Society (LEOS), Boston Chapter, winning the LEOS Chapter-of-the-Year Award during 1994–1995. He is an Associate Editor for IEEE JOURNAL OF QUANTUM ELECTRONICS and Vice President of LEOS for membership and regional activities – Americas. He has been selected as a LEOS Distinguished Lecturer for 2005–2007 and Australian Research Council Nanotechnology Network Distinguished Lecturer for 2007. He was the recipient of the National Science Foundation Research Initiation Award in 1993, United Nations TOKTEN award in 1995 and 1996, and both the National Science Foundation CAREER and Office of Naval Research Young Investigator Awards in 1996. He was the former Chair of the IEEE/LEOS technical committee on Photodetectors and Imaging and currently, the Chair of IEEE/LEOS Nanophotonics committee.