

# A Demonstration of High-throughput Immunoassay and Small Molecule Binding on Protein Microarrays with SPR Microscopy

Gibum Kim, Lei Jiang, Pradipsinh K. Rathod, Charles T. Campbell, Akiko Nishimoto\*, and Vincenzo Casasanta\*

Department of Chemistry, University of Washington, Seattle, Washington, 98195-1700

\*Lumera Corporation., 19910 North Creek Parkway, Bothell, WA 98011

## ABSTRACT

The use of Surface Plasmon Resonance (SPR) for the detection of biomolecular binding events at the nanometer scale is of great utility when expanded into microarray density formats.<sup>1-3</sup> Protein microarray analysis is particularly well suited for such methodology. In this work we demonstrate a high-throughput, quantitative, label-free immunoassay by employing microarray techniques in combination with SPR microscopy. Antibody microarrays with various immunoglobulin proteins, including human immunoglobulin subclasses were printed on functionalized gold-coated glass slides and the binding specificity of each antibody toward antigens confirmed that the activity of the antibodies remained after microarray fabrication. Gold-coated glass slides presenting amine reactive moieties were prepared to facilitate protein microarray printing. Antigen microarrays were printed similarly with various allergens including human serum albumin as well as bovine serum albumin. As a demonstration of quantitative, low molecular weight drug-molecule screening in real-time with SPR microscopy, the binding kinetics of 1,400 Da biotinylated peptides were monitored on streptavidin (SA) spots attached on biotinylated BSA arrays on functionalized gold-coated glass slides. A spot density of up to 240 120- $\mu\text{m}$  spots was generated using conventional robotic microspotting. Application of antibody/antigen microarrays with SPR microscopy allows for the simultaneous monitoring of immunoglobulin protein binding kinetics over the whole array (allergens or antibodies), at a time resolution of one second. The ability of microarray-based SPR to quantitatively monitor antigen-antibody binding kinetics in "real-time" without labels with a sensitivity of 1.2 ng/cm<sup>2</sup> (<0.5 pg per spot) for most proteins<sup>1</sup> allows it to become a complementary tool in support of the multi-step and labor-intensive ELISA method.

**Keywords:** proteomics, microarray, SPR, immunology

## 1 BACKGROUND

As progress in proteomics continues, there will be greater need for label-free detection schemes for protein microarrays. A powerful method capable of detecting

molecular binding events at the nanometer scale is Surface Plasmon Resonance (SPR). By using a CCD array detector in the Kretschmann configuration a SPR microscope can be constructed. This detection scheme provides for the simultaneous monitoring of binding events at different regions across patterned sensing areas<sup>1-3</sup> with a resolution down to  $\sim 4 \mu\text{m}$ .<sup>3</sup> For SPR Microscopy, the spatial contrast due to the difference in reflected light intensity over a heterogeneous area is measured using a b/w CCD camera whose signal is converted to real time changes in molecular surface coverage as described in ref. <sup>6</sup>

In most SPR applications the biosensor surface consists of a gold (Au) film of 40-60 nm thickness for which the plasmon is generated by incident light. Immobilization of the biomolecule of interest to this Au surface is therefore of prime importance in order to detect the refractive index changes indicative of molecular binding. Various functionalization strategies have been reported to immobilize biomolecules on these Au-coated glass surfaces for SPR detection.<sup>1-5</sup> Earlier reports employed biotinylated gold surfaces for further surface attachment using a linker molecule of SA, a protein which binds biotin as a ligand in four sites.<sup>1,4,6</sup> In turn the SA allows further attachment of a variety of other biotinylated molecules. For example, biotinylated DNA strands have been attached on mixed self-assembled monolayers of biotinylated alkylthiol with ethylene glycol functionalized alkylthiol by using SA as a linker.<sup>1,3,5</sup> In this same vein, Au surfaces have been modified with amine-terminated alkylthiols to immobilize amine-terminated DNA after additional crosslinking.<sup>5</sup>

The method used here for covalent attachment of proteins on the SPR active surface is proprietary but may be described at the conference. A brief schematic of the method is shown in Fig. 1. A linker layer which terminates with functional groups which can covalently bond to organic amines is first prepared and dried. Then the proteins are patterned to covalently react with this linker layer using microdroplets from a robotic spotter.

Proteomic systems which provide models to study the latitude of SPR microscopy are exhibited in the immunoglobulins, and have been studied here. Antibody-antigen binding reactions form a rational basis to evaluate the efficiency, power, and utility of array-based SPR techniques since abundant reference data is known from the

ELISA technique. As array SPR methodology progresses, such models can be extended into other applications involving protein-protein and protein-DNA interactions which will be of utility for protein network mapping, drug discovery, and toxicology.

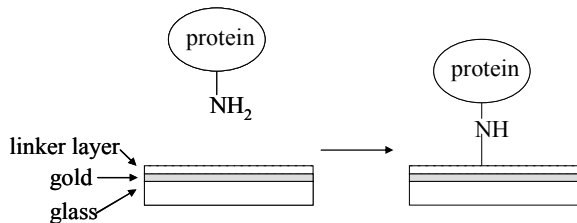


Figure 1. Protein immobilization step

In this paper we microspot protein solutions and immobilize to produce protein microarrays on functionalized Au biosensors and study a series of antibody-antigen systems by SPR microscopy. The label-free detection of molecules much smaller than proteins ( $< 2\text{kDa}$ ) is an attractive frontier for array SPR analysis since there may be applications in real-time quantitative screening of drug molecules and the like. To that end we also demonstrate the SPR detection of small biotinylated peptides (1400 Da.) binding to immobilized SA in a protein microarray format.

## 2 EXPERIMENTAL

### 2.1 Surface Modification

The SPR-active surfaces used in this work were prepared on Au coated glass slide surfaces. The gold surfaces as received (Au-coated SF-10 glass slides from Gentel Biosurface Madison, WI) were first cleaned with a piranha solution at  $80^\circ\text{C}$  for 15 seconds, rinsed with distilled ( $18\text{ M}\Omega\cdot\text{cm}$ ) water and absolute ethanol, and dried in  $\text{N}_2$ . They were then functionalized with amine-reactive organic headgroups in a proprietary linker layer.

### 2.2 Protein Microarray Fabrication

Protein microarrays were solution spotted onto these SPR active slides using a robotic pin microarrayer. The protein solutions were prepared by dissolving proteins in spotting buffer in 5% DMSO. The protein concentrations of these spotting solutions ranged from 0.5 mg/ml to 2 mg/ml and the arrayer delivery volume ranged from 0.3 to 0.6 nl. Delivery destinations were programmed to form spots within a 4mm x 5mm area to produce up to 240 separate sample spots. Immobilization of the deposited proteins was achieved by covalent coupling between the functionalized SPR active slide and the protein, forming the

stable linkage shown in Fig. 1. The arrays were rinsed with distilled water and dried in  $\text{N}_2$ .

## 3 RESULTS AND DISCUSSION

### 3.1 Protein-Protein SPR Detection

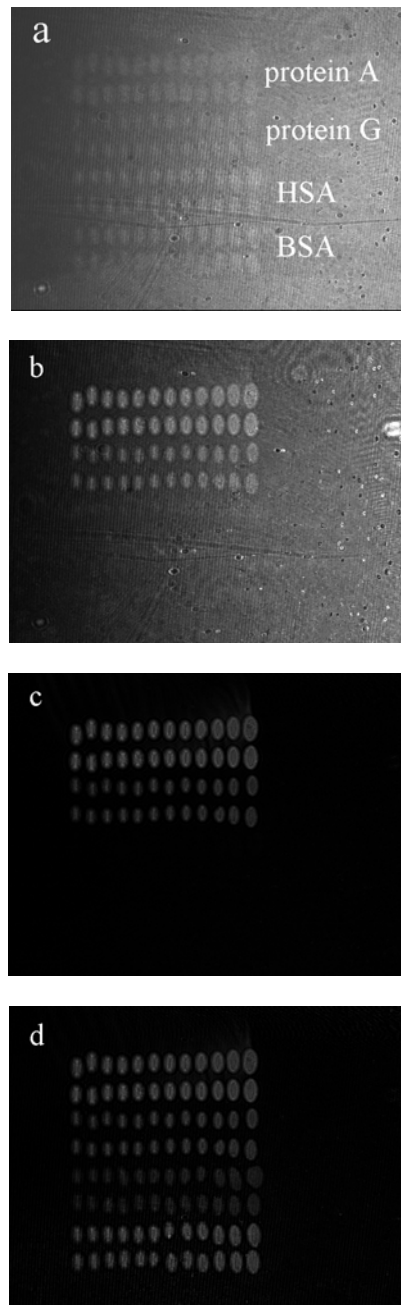


Figure 2. SPR microscope images of (a) protein microarray, before exposed to antibodies, protein microarray after exposed to 0.1 mg/ml human IgG in PBS, and difference images after exposed to 0.1 mg/ml human IgG in PBS (c) and 0.1 mg anti-BSA IgG in PBS (d). Image (b), (c) and (d) were captured after surface excess antibodies were rinsed with pure PBS.

The SPR microscope images shown in Fig. 2 are grey-scale representations of the reflected light intensity at a high-contrast angle (see ref 6) for an 8 x 12 array of spots within an area of 3.6 mm × 2.4 mm. The spots in this particular experiment were varied in size from ~160 to ~300 μm diameter and offer redundant samples as follows:

- Rows 1 & 2 (top): spotted with protein A
- Rows 3 & 4 (top middle): spotted with protein G
- Rows 5 & 6 (bottom middle): spotted with human serum albumin (HSA)
- Rows 7 and 8 (bottom): spotted with bovine serum albumin (BSA)

The protein A and protein G spots serve as active binding sites for the analytes of interest, and the HSA and BSA present non-binding control sites. The top figure (2a) shows the array reflectivity prior to any binding reactions. Fig. 2b shows the protein microarray image after exposure to 0.1 mg/ml of human immunoglobulin G (IgG) dissolved in 100 mM phosphate buffered saline (PBS) solution and subsequently rinsed with the same PBS alone. Proteins A and G show increased binding induced reflectivity as expected. This binding can be quantified using basic image analysis involving integration and background subtraction. Such an operation is displayed in Fig. 2c which is the difference image between Figures 2a and 2b. The binding surface densities can be calculated from these net reflectivity increases (RI), following the procedure outlined in ref. 6 and are:

- Human IgG on Protein A:  $22.9 \pm 1.5 \text{ ng/cm}^2$  (derived from  $27.1 \pm 1.8 \text{ \%RI}$ )
- Human IgG on Protein G:  $14.4 \pm 1.1 \text{ ng/cm}^2$  (derived from  $17.3 \pm 1.3 \text{ \%RI}$ )
- Human IgG on HSA:  $0.5 \pm 0.1 \text{ ng/cm}^2$  (derived from  $0.6 \pm 0.1 \text{ \%RI}$ )
- Human IgG on BSA:  $0.2 \pm 0.1 \text{ ng/cm}^2$  (derived from  $0.4 \pm 0.1 \text{ \%RI}$ )

The image of Fig. 2d shows the same type of difference image for the case in which the microarray is exposed to anti-BSA IgG (0.1 mg/ml) in PBS solution. Here the anti-BSA IgG binds to all four of the proteins with much less specificity than the human IgG experiment. During the protein binding SPR experiments the CCD output can be monitored in real time such that their kinetics can be characterized (ref. <sup>1</sup>). An example of this is shown in Fig. 3. For the experiments described above, the average reflected light intensity of each protein spot of Fig. 2 was collected at a time resolution of 1 s. The conversions to binding ratio versus time are plotted in Fig. 3 for the human IgG (top) and anti-BSA IgG (bottom) experiments. These values show the very selective binding of human IgG to protein A and protein G, compared to the almost negligible binding of human IgG to the control spots (HSA and BSA). The saturation stoichiometries observed here are in good

agreement with previous studies of the relative ratios of these binding pairs.<sup>7-9</sup>

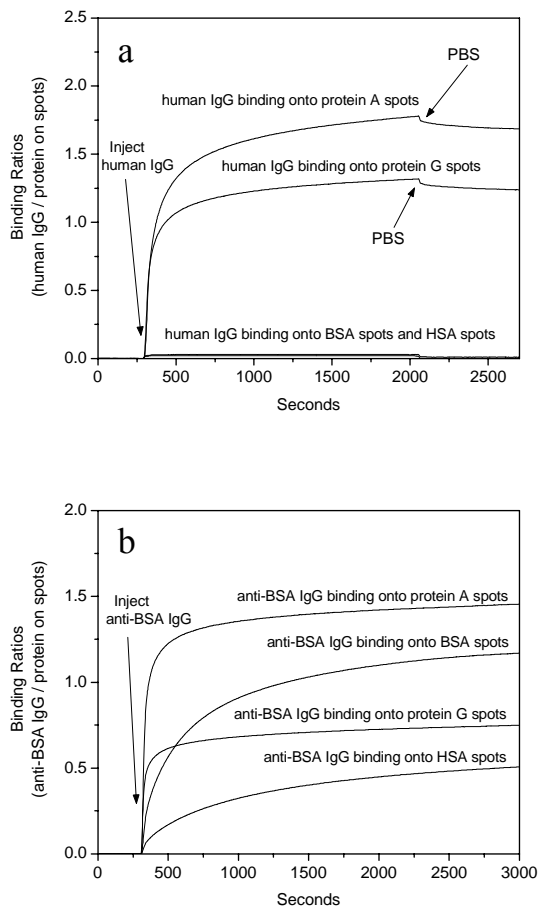


Figure 3. The binding kinetics of human IgG and anti-BSA IgG onto protein A, protein G, HSA, and BSA spots. The difference in the reflected light intensity on each protein spot on the microarray was converted to the surface coverage as a function of time.

### 3.2 Small Molecule Detection

The opportunity to perform high-throughput, label-free detection of small molecules by SPR microscopy is explored here using a biotinylated peptide of molecular weight of 1400 Da. Biotinylated BSA was spotted from protein solution (1 mg/ml in spotting solution with 5 % DMSO) on the functionalized SPR active surface. SA was bound onto the biotinylated BSA as a linker molecule for the attachment of this biotinylated peptide. The binding of SA onto the biotinylated BSA spots is shown in Fig. 4a. Finally, the binding of the biotinylated peptide test molecule to these SA spots is clearly observable, as shown in Fig. 4b.

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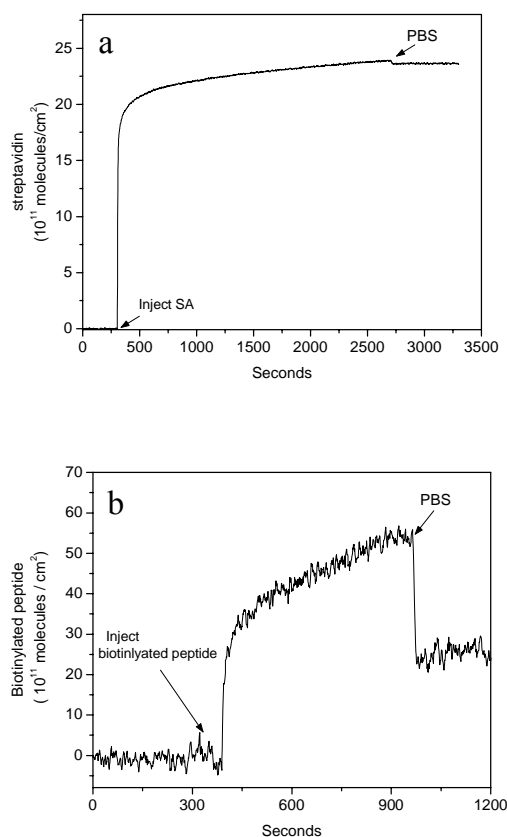


Figure 4. Small molecule SPR binding curves. a) The baseline was established by introducing running buffer onto the array surface, then SA in running buffer. As the SA adsorption approached saturation, the array was rinsed with buffer. b) Binding curve upon injection of the 1400 Da biotinylated peptide test molecule in running buffer.

## 4. CONCLUSION

Assays performed in this work have proven that protein microarrays can be applied in combination with SPR microscopy to create a very effective immunoassay technique with quantitative parallel analysis in real time, with one sec time resolution. This can provide crucial information regarding biological interactions which otherwise can not be observed with the traditional ELISA technique. The high sensitivity of SPR microscopy also showed possibility of detecting drug molecules binding to immobilized protein arrays, as shown in Fig 4. Further studies focused on the application of new microarray techniques for protein-protein interaction as well as binding of small drug molecules (~1000 Da.) will be presented in a complementary publication.