Advancing Label-free Microarray Immunoassays with Protein Capture of Antibodies

A Demonstration by Oblique Incidence Reflectivity Difference (OI-RD) Ellipsometry

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Abstract

Oblique Incidence Reflectivity Difference (OI-RD), a system developed by Zhu et al, is a versatile, high-throughput, label-free method for probing reactions of biological molecules printed on standard substrates. However, its sensitivity range is currently not as developed as that of label-based techniques. The effectiveness of using recombinant fusion protein A/G to immobilize antibodies on a glass substrate and amplify reaction signals in OI-RD was tested with generic micro-immunoassays. Results suggest that the protein capture method for antibody analysis can amplify OI-RD signals such that those from nanomolar concentrations are still distinct. It was also more generally implicated that protein capture could be used to purify and react antibodies from cell supernatant *in situ*. But, the technique was found to reduce the binding affinity of the antibodies relative to samples directly printed on substrate.

I. Introduction

Microarrays, two-dimensional arrangements of micrometer-sized spots of immobilized biological molecules, have revolutionized standards for gathering information in biology and medicine [1, 2]. Compact size and storage, small reagent volume requirements, and capacity for automated production and high-throughput analysis are among the distinct advantages of the microarray paradigm. In their utility, microarrays have helped further our understanding of a myraid of biological molecules, but of particular interest to our study are protein microarrays.

Many protein microarrays utilize antibodies because of their specificity and versatility in binding capabilities [3]. Thus using antibody microarrays in micro-immunoassays to detect and quantify target compounds in complex mixtures has been a successful technique. However, most commonly used methods are label-based; that is, they involve attaching a label molecule to the molecule to be detected. And, although traditional label-based assays have well established technology and sensitivity ranges, effective label-free techniques are desirable supplements because covalent molecular modification, especially for proteins, may have unpredictable kinetic effects [4, 5].

The oblique incidence reflectivity difference (OI-RD) microscope is a scanning ellipsometer capable of high-throughput, label-free analysis of microarray reactions [6–10]. Although its sensitivity may not be as developed as that of fluoresence microscopes, its versatility as a label-free technique utilizing standard glass microchips makes OI-RD a very practical supplement for label-based methods. In terms of micro-immunoassays, OI-RD can be used to scan for ligand binding directly or through immuno-sandwich reactions. Because the microscope functions by detecting changes in polarization state of light reflected from the thin film of biological molecules, no fluorophores or other labeling agents are required at all.

Protein A/G is a recombinant fusion protein of naturally occuring proteins A and G, bacterial antibody binding proteins. Both proteins bind to areas in the constant domains of the Fc regions of immunoglobulins, thus their ability to capture antibodies is often exploited to purify complex immunoglobulin-containing solutions [11]. This ability also makes it a candidate for a microarray scaffolding molecule which may be able to stabilize immobilized antibodies and increase OI-RD signal amplitudes.

Thus the motivation for our study is to test the utility of protein A/G as a method for extending the sensitivity range of the OI-RD microscope without modifying microscope hardware, evaluate the effects of protein A/G capture on antibody binding affinity, and to explore its potential for usage in *in situ* purification and reaction of antibodies.

II. Optical Setup and Theory

In general, ellipsometry is an optical technique for gathering information about surfaces by measuring changes in polarization state (i.e. magnitude and phase) of elliptically polarized light reflected from the surface [12]. OI-RD is a type of polarization-modulated nulling ellipsometry that directly measures the differences in fractional changes in reflectivity between p- and s-polarized components of light. At an oblique angle of incidence, the polarization states of p- and s-polarized light change disproportionately in response to alterations on the surface of reflection and thus can provide information about reactions occuring on a thin film on a substrate [13].

Let the reflectivities for the p- and s-polarized components of a monochromatic light at wavelength λ on a bare substrate surface be given by $r_{p0} = |r_{p0}|e^{i\Phi_{p0}}$ and $r_{s0} = |r_{s0}|e^{i\Phi_{s0}}$, respectively. And, let $r_p = |r_p|e^{i\Phi_p}$ and $r_s = |r_s|e^{i\Phi_s}$ be the respective reflectivities of the substrate to p- and s-polarized light when it is covered with a thin film of molecules of thickness d. The fractional changes in reflectivity are then given by $\Delta_p \equiv \frac{r_p - r_{p0}}{r_{p0}}$ and $\Delta_s \equiv \frac{r_s - r_{s0}}{r_{s0}}$. Thus the difference in fractional changes in reflectivities is given by $\Delta_p - \Delta_s$. When $\Delta_p - \Delta_s$ is small, its real part, $Re\{\Delta_p - \Delta_s\} \approx \frac{|r_p|-|r_{p0}|}{|r_{p0}|} - \frac{|r_s|-|r_{s0}|}{|r_{s0}|}$, is the differential change in magnitude; its imaginary part, $Im\{\Delta_p - \Delta_s\} \approx (\Phi_p - \Phi_{p0}) - (\Phi_s - \Phi_{s0})$, is the differential change in phase. In terms of experimentally measurable quantities, Zhu and coworkers have shown that

$$I_\Omega \approx Im\{\Delta_p - \Delta_s\} \approx -\left[\frac{4\pi\sqrt{\varepsilon_s}\cos\theta}{(\varepsilon_s - \varepsilon_0)(\cot^2\theta - \varepsilon_0/\varepsilon_s)}\right] \times \left[\frac{(\varepsilon_d - \varepsilon_s)(\varepsilon_d - \varepsilon_0)}{\varepsilon_d}\right]\left(\frac{d}{\lambda}\right),$$

where θ is the angle of incidence and ε_s, ε_d, and ε_0 are the dielectric constants of the substrate, the thin film, and the ambient space, respectively.

A schematic of an OI-RD microscope with a prism is shown in Figure 1. There are a few variations on the OI-RD microscope, but the concept behind each is essentially the same. Light from a plane polarized monochromatic source is passed through a photoelastic modulator, which causes the beam to fluctuate between p- and s-polarization at a certain frequency Ω. The polarization-modulated light encounters a phase shifter that creates an adjustable phase Φ_{ps} between the p- and s-polarized components. The light is then focused onto the microchip containing the microarray to be analyzed at an incident angle θ. Afterwards, the reflected beam is refocused through a polarizing analyzer and the intensity $I(t)$ of the resultant light beam is characterized by a photodiode detector. $I(t)$ contains a number of harmonics of frequency Ω, and the first harmonic I_Ω is detected with a lock-in amplifier. And, an automated fluidics system is responsible for the timely influx and efflux of buffer and reaction solutions into the reaction chamber(s).

OI-RD is capable of both taking reaction end-point images and of recording real-time kinetics curves of reactions between solution phase probes and immobilized targets. An image is obtained by scanning the beam across the microchip at a certain scanning resolution. End-point measurements can be made by comparing images of a microchip before and after exposure to reactants. On the other hand, real-time kinetics curves can be acquired by scanning all or a selected subset of points on a microarray rapidly as reactants are flowed through a reaction chamber.

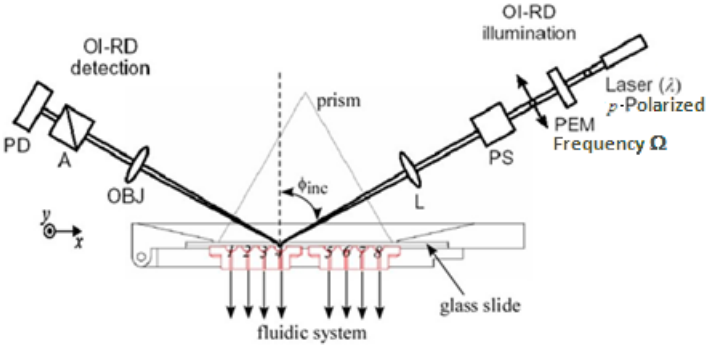

Figure 1: Schematic of an OI-RD microscope with a prism for total internal reflection. The microchip is mounted in a metal clasp, the reaction chamber, which holds the microarrays in position against the fluidics system. PEM, photoelastic modulator for modulating polarization; PS, phase shifter; L, focusing lens; OBJ, objective lens for facilitating detection; A, polarization analyzer; PD, photodiode detector.

III. Experimental Methods

A microarray containing bovine serum albumin (BSA), protein A/G, and goat anti-rabbit polyclonal antibodies was fabricated using a contact printer printing on epoxy-coated glass slides. As BSA is unreactive to any proteins used in the experiments, it serves as a control substance. The printing concentration of BSA was 0.5 mg/mL. Both the protein A/G and the polyclonal goat anti-rabbit antibodies were printed in concentrations of 0.5 μM, 1 μM, 2 μM, 4 μM, and 8 μM.

The fluidics routine used at each step totaled 120 minutes. First was a 30 minute baseline period during which buffer solution, 1X PBS, was flowed through the reaction chamber. Then, a rapid injection of probe solution exchanged the aqueous environment in the reaction chamber from buffer to probe, and this was followed by a period of slow flow of probe solution, totaling 30 minutes. This period is the *association phase*. And finally, a rapid injection of buffer solution exchanged the aqueous environment from probe to buffer and was followed by a slow washing of the microarray with buffer solution for 60 minutes. This period is the *dissociation phase*.

A. Signal Amplification and Binding Affinity

After the microchip was mounted in the OI-RD microscope, it was washed with 1X PBS to remove unbound proteins and buffer salt precipitates.

Then, a solution of 0.5 mg/mL BSA in 1X PBS was flowed through the reaction chamber as a blocking agent. Unreacted epoxy groups from the microchip coating, especially in the unprinted regions, were blocked with BSA to prevent subsequent reagents from nonspecifically reacting with the substrate.

Once the glass slide had been blocked with BSA, a 40 nM solution of polyclonal goat anti-rabbit antibodies in 1X PBS was flowed into the reaction chamber to be captured by the printed protein A/G spots.

Next, rabbit IgG, an antigen for the printed and captured polyclonal goat anti-rabbit antibodies, was flowed into the reaction chamber. To measure the antibody binding affinities, the experiment was conducted over a range of concentrations of rabbit IgG in 1X PBS: 10 nM, 40 nM, and 160 nM.

B. *in situ* Purification and Reaction

The microchip was mounted into the OI-RD microscope and washed with 1X PBS.

Then, the glass slide was blocked with 0.5 mg/mL BSA in 1X PBS.

Next, a complex serum containing rabbit anti-human polyclonal antibodies was flowed into the reaction chamber to be captured by printed protein A/G.

Finally, a 80 nM solution of human IgG in 1X PBS was flowed into the reaction chamber to react with the captured rabbit anti-human polyclonal antibodies.

End-point images and real-time kinetics curves were recorded for every step to characterize reactions.

IV. Results and Discussion

A. Signal Amplification and Binding Affinity

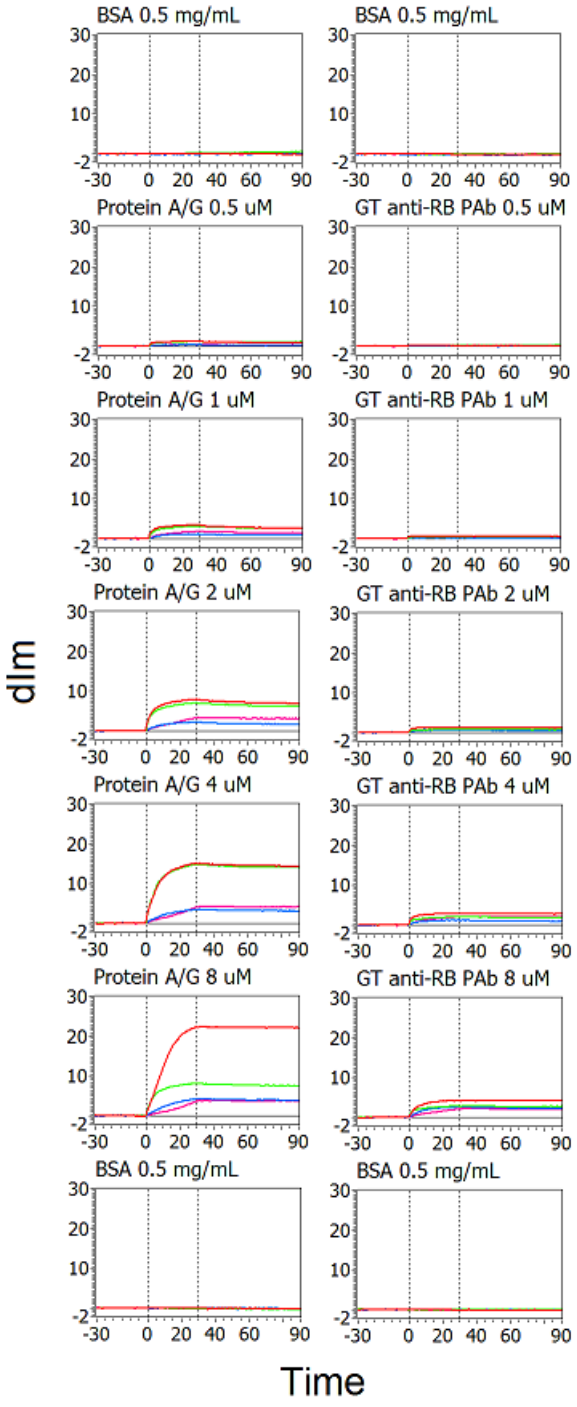

Figure 2: A sample of real-time kinetics curves for a reaction between captured and printed polyclonal goat anti-rabbit antibodies and rabbit IgG at 40 nM (blue and purple) and 160 nM (red and green). The end of the baseline period and the beginning of the association phase are denoted by the dotted line at t = 0 minutes; the end of the association phase and beginning of the dissociation phase are denoted by the dotted line at t = 30 minutes.

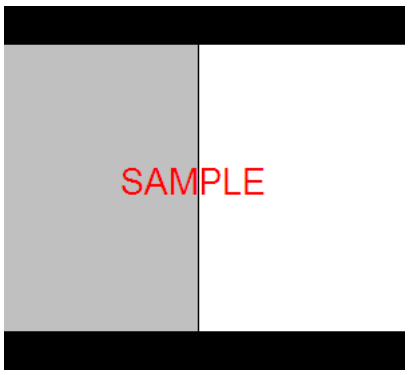

Figure 3: A binding affinity map of the printed microarray. A higher binding affinity is represented by a brighter map square. Binding affinities were determined by applying a one-to-one fitting model to the set of curves obtained from reactions of captured and printed polyclonal goat anti-rabbit antibodies with 40 nM and 160 nM solutions of rabbit IgG.

An excerpt of the set of curves obtained from reactions between captured and printed polyclonal goat anti-rabbit antibodies and solutions of rabbit IgG at 40 nM and 160 nM is shown in Figure 2. The final curves were generated by subtracting curves from control spots in the unprinted regions from the curves of printed spots as to correct for any signal drift in the system. Although the amplitude of signals generated by reactions at the same probe concentration may not be homogenous within this data set for reasons left to experimental variations, it is a very clear and consistent trend that the signal amplitude of protein A/G captured antibodies is significantly greater than that of antibodies directly printed on the substrate. It should also be mentioned that even though the data generated from reactions with 10 nM probe solution were omitted due to experimental errors which rendered the data incompatible with an *en masse* calculation of binding affinities, the signal amplification trend was noticed within the usable data points.

Figure 3 demonstrates a map of the microarray representing the mean binding affinity of each target point. The binding affinity was estimated using a one-to-one binding fit model which was sufficient to describe the general behavior of association and dissociation given by the data. In Figure 2, it can be noted that the curvature of the signals generated at different probe concentrations for protein A/G captured antibodies during the association phase varied between semi-linear and logarithmic. The variation is most noticable for greater printing concentrations of protein A/G, and the curvature becomes more consistent at lower printing concentrations. This is again most likely due to experimental margins in the fabrication of the microarrays. Although this does affect the specific values of binding affinity calculations, a general trend was still noted. While the signal amplitude was magnified by the protein A/G capture of antibodies, the map indicates that the binding affinities of captured antibodies was actually lowered in comparison to antibodies printed directly on the substrate.

B. *in situ* Purification and Reaction

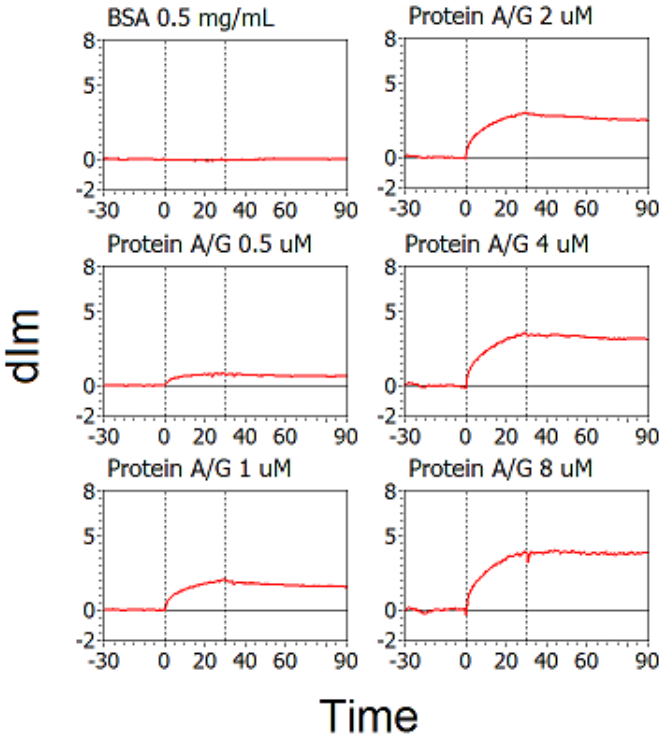

Figure 4: A sample of real-time kinetics curves for a reaction between polyclonal rabbit anti-human antibodies captured by protein A/G from a complex serum and human IgG at 80 nM. The end of the baseline period and the beginning of the association phase are denoted by the dotted line at t = 0 minutes; the end of the association phase and the beginning of the dissociation phase are denoted by the dotted line at t = 30 minutes.

Figure 4 shows an excerpt of the set of curves from a reaction between polyclonal rabbit anti-human antibodies captured from a complex serum by protein A/G and human IgG at 80 nM. The signals are clean and distinct, which suggests that using protein A/G to capture antibodies out of solution before reacting them de-necessitates purified antibody solutions. That is, antibodies can be purified *in situ* from a complex mixture, given that there is only one type of desired antibody in solution, before a reaction with an antigen solution.

V. Conclusion

Oblique incidence reflectivity difference ellipsometric microscopy is an effective technique for high-throughput, label-free microarray analysis. Although it is currently not as sensitive as fluoresence-based detection, it has been demonstrated that there are "soft" methods of improving sensitivity range without changing the microscope hardware. Using protein A/G as scaffolding molecules for antibody microarrays is one such example. The results of these experiments suggest that protein A/G can improve signal amplitudes, at the apparent cost of binding affinity, and can purify mixed solutions *in situ*. This suggests that smaller concentrations of often expensive antibodies can be used to obtain meaningful signals and that highly purified antibody solutions may not be required for effective reactions. Moreover, the combined conclusions address the possibility of a more cost-efficient microarray fabrication technique in which an array of protein A/G is printed over with a complex solution of, for example, hybridoma cell supernatant to generate a workable antibody microarray. Future work may include experiments to assess that possibility and to explore and characterize the mechanistic nature of the signal amplification and binding affinity dampening.

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