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2010 J. Opt. 12 095301

(http://iopscience.iop.org/2040-8986/12/9/095301)

J. Opt. **12** (2010) 095301 (5pp) doi:10.1088/2040-8978/12/9/095301

Detection of the specific binding on protein microarrays by oblique-incidence reflectivity difference method

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Received 8 June 2010, accepted for publication 28 July 2010
Published 6 September 2010
Online at stacks.iop.org/JOpt/12/095301

Abstract
The specific binding between Cy5-labeled goat anti-mouse Immunoglobulin G (IgG) and mouse IgG with a concentration range from 625 to 10^4 μg ml^{-1} has been detected successfully by the oblique-incidence reflectivity difference (OI-RD) method in each procedure of microarray fabrication. The experimental data prove that the OI-RD method can be employed not only to distinguish the different concentrations in label-free fashion but also to detect the antibody–antigen capture. In addition, the differential treatment of the OI-RD signals can decrease the negative influences of glass slide as the microarray upholder. Therefore the OI-RD technique has promising applications for the label-free and high-throughput detection of protein microarrays.

Keywords: oblique-incidence reflectivity difference, protein microarrays, label-free detection

1. Introduction

A systemic understanding of the structure, functionality and regulation of proteins challenges the proteomic research in this information-rich age of whole-genome biology. In the quest to learn how a number of proteins interact with each other under a wide range of conditions, protein microarray technology has emerged as a promising approach, which allows the simultaneous determination of large quantities of parameters from a minute amount of a sample within a single experiment in a rapid, parallel and high-throughput fashion [1, 2]. For the high-throughput detection of microarrays, fluorescence labeling is by far the most preferred because of its inherently high sensitivity and large dynamic range [3–5]. However, extrinsic fluorescent labels may have an unpredictable and profound influence on the physical and chemical properties of protein molecules. In addition, the cost as well as the efficiency of the fluorescence labeling process can be undesirable *a priori*. Circumventing these obstacles in label-based systems, it is thus reasonable to explore other label-free detection methods with adequate sensitivities to complement fluorescence-based detection.

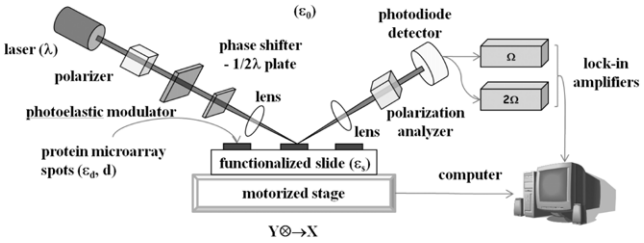

Figure 1. The layout of the OI-RD system for the detection of protein microarrays. Laser: a 7 mW He–Ne p-polarized laser. The microarray is mounted on a motorized stage that can be driven along the *X* and *Y* directions, respectively.

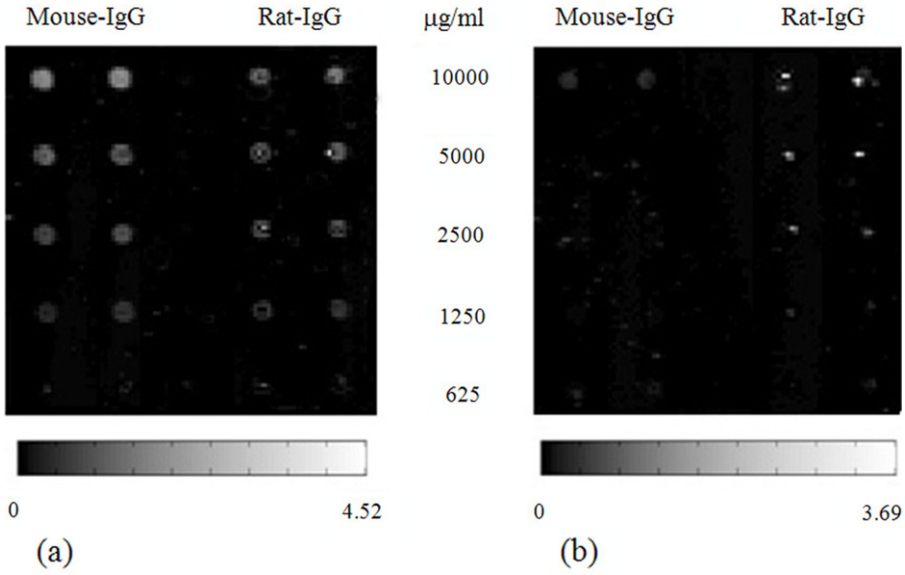

Figure 2. The intensity images of OI-RD two-dimensional scan for the washed mouse and rat IgG microarray. (a) $\mathrm{Im}\{\Delta_p - \Delta_s\} \times 100$ image; and (b) $\mathrm{Re}\{\Delta_p - \Delta_s\} \times 100$ image.

Oblique-incidence reflectivity difference (OI-RD), as a label-free and sensitive detection technique, was applied to *in situ* monitoring of the growing process of oxide thin films in our early work based on the change of optical reflectivities between the two orthogonal components of s- and p-polarized light from a surface [6, 7]. Our experimental results showed that the detection sensitivity for the difference in the reflectivity change $\Delta R/R$ between s- and p-polarized light can reach 2×10^{-5} [7]. Therefore, the OI-RD technique can be employed in the label-free detection of biological microarrays since the binding of biological molecules could lead to changes in the optical dielectric constant. Previously, Zhu *et al* reported the detection of 60-base oligonucleotides [8] and protein microarrays [9] by the imaginary part of the OI-RD signal. Moreover, we have reported the employment of the OI-RD method for the detection of 20-base oligonucleotides [10]. In the present work, we have detected the specific binding between mouse Immunoglobulin G (IgG) at different concentrations and Cy5-labeled mouse IgG (anti-mouse IgG) using both the imaginary and real parts of OI-RD signals simultaneously. A comparison with fluorescent detection demonstrates that the OI-RD method is a promising technique for microarray assay resulting from its successful detection of antibody–antigen capture as well as the label-free distinguishment of diverse concentrations.

2. Experimental details

A typical OI-RD setup for the detection of protein microarrays is shown in figure 1. Similar to those employed for the detection of oxide film growth [6], a p-polarized He–Ne laser beam with $\lambda = 632.8$ nm passes through a photoelastic modulator (PEM 90) that induces the laser beam to oscillate between p- and s-polarization at a frequency $\Omega = 50$ kHz. A phase difference between p- and s-polarized components is introduced by a phase shifter. Then the light beam is focused on the microarray surface at an incident angle of 60°. The reflected beam passes through a polarization analyzer and is detected by a silicon photodiode. Finally the first $I(\Omega)$ and second harmonics $I(2\Omega)$ of the reflected beam intensity are monitored by two digital lock-in amplifiers.

Briefly, let $r_{p0} = |r_{p0}| \exp(i\varphi_{p0})$ and $r_{s0} = |r_{s0}| \exp(i\varphi_{s0})$ denote the respective reflectivity from the bare microarray surface (no protein molecules) for p- and s-polarized light respectively, and $r_p = |r_p| \exp(i\varphi_p)$ and $r_s = |r_s| \exp(i\varphi_s)$ be the respective reflectivity from the protein molecules on the surface. The changes in reflectivity are defined as $\Delta_p = (r_p - r_{p0})/r_{p0}$ and $\Delta_s = (r_s - r_{s0})/r_{s0}$ for p- and s-components respectively. Then, the OI-RD signal, the difference of fractional reflectivity change, is $\Delta_p - \Delta_s$. In the experiment, we directly measured $I(\Omega)$ and $I(2\Omega)$, which are proportional to the imaginary $\mathrm{Im}\{\Delta_p - \Delta_s\}$ and real parts $\mathrm{Re}\{\Delta_p - \Delta_s\}$ respectively. At the beginning, the laser beam is focused on the bare microarray surface on the condition that $\mathrm{Im}\{\Delta_p - \Delta_s\}$ and $\mathrm{Re}\{\Delta_p - \Delta_s\}$ are zeroed by adjusting the phase shifter and the analyzer respectively so as to improve the signal-to-noise ratio. Afterward the intensities of both $\mathrm{Im}\{\Delta_p - \Delta_s\}$ and $\mathrm{Re}\{\Delta_p - \Delta_s\}$ are recorded when the microarray stage is scanned in two dimensions.

An aldehyde-coated glass slide (CEL Associates, TX) was chosen as the microarray substrate. Mouse and rat IgG were selected as the targets and Cy5-labeled goat anti-mouse IgG as the probe (all the IgG was purchased from KPL Inc, USA). We fabricated the IgG microarrays following the conventional procedure described in [11]. At first, using a SpotBot®2 complete contact-printing microarrayer (Arrayit Corporation, Sunnyvale, CA), the mouse and rat IgG were separately printed in duplicate with a concentration decreasing from top to bottom in a range from 10^4 to 625 $\mu g\,ml^{-1}$ by a factor of 0.5 respectively as shown in figures 2 and 3. Meanwhile, the immobilized mass of IgG molecules ranges from 11 to

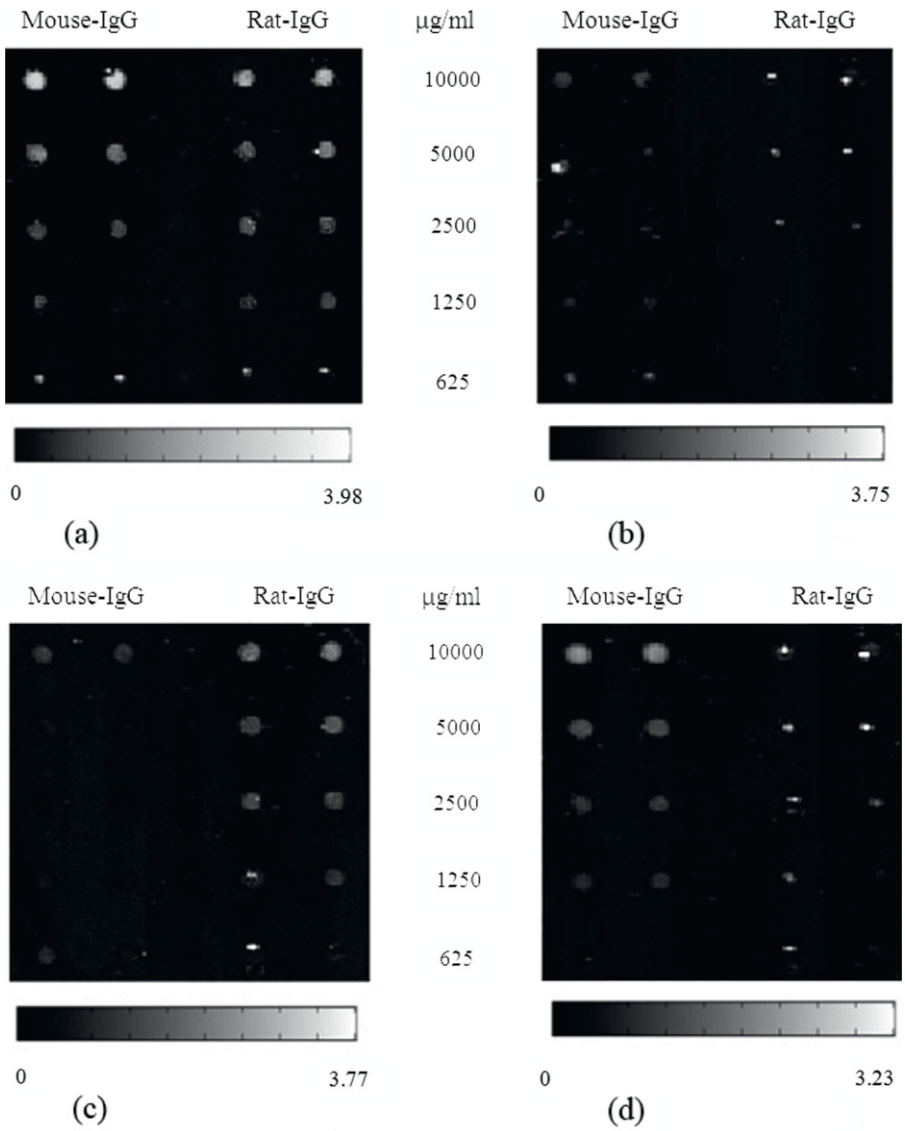

Figure 3. 2D scan images of the OI-RD and fluorescence scan before and after specific binding between mouse IgG and Cy5-labeled anti-mouse IgG. (a) $\mathrm{Im}\{\Delta_p - \Delta_s\}$ image after blocking; (b) $\mathrm{Re}\{\Delta_p - \Delta_s\}$ image after blocking; (c) $\mathrm{Im}\{\Delta_p - \Delta_s\}$ image after specific binding; (d) $\mathrm{Re}\{\Delta_p - \Delta_s\}$ image after specific binding; (e) the differential $\mathrm{Im}\{\Delta_p - \Delta_s\}$ image obtained by subtracting (a) from (c); (f) the differential $\mathrm{Re}\{\Delta_p - \Delta_s\}$ image obtained by subtracting (b) from (d); (g) the fluorescent scan image after specific binding.

0.688 ng because one sample spot contains about 1.1 nl solution in our experiment. The center to center separation between sample spots with an average diameter of 135 μm is about 750 μm. Second, the IgG microarrays were washed with 1 × PBS for 5 min and $\mathrm{ddH_2O}$ for 5 min and spun dry in a minicentrifuge (Labnet International, Woodbridge, NJ) at 4800 rpm for 45 s sequentially in order to remove the excess unbound protein and buffer the salt precipitates. Third, the biochips were blocked with 1% glycine dissolved in 1 × PBS for 1 h at room temperature to quench the intact aldehyde groups on the surface. Finally, 20 $\mu\mathrm{g\ ml^{-1}}$ Cy5-labeled goat anti-mouse IgG diluted with 1% glycine/1 × PBS was reacted with the IgG microarrays at room temperature for 1 h in a humidity chamber. After rinsing with PBST for 5 min, 1 × PBS for 5 min and $\mathrm{ddH_2O}$ for 5 min sequentially, the slides were dried by minicentrifuge as mentioned.

The microarrays were detected after each procedure of fabrication by the OI-RD method respectively, and eventually detected after antibody–antigen capture by both OI-RD and fluorescent scan (LuxScan 10 K, CapitalBio, China).

3. Results and discussions

Figures 2(a) and (b) show the intensity images of $\mathrm{Im}\{\Delta_p - \Delta_s\}$ and $\mathrm{Re}\{\Delta_p - \Delta_s\}$ by OI-RD two-dimensional (2D) scan for a washed IgG microarray before blocking. It is obvious that the image of $\mathrm{Im}\{\Delta_p - \Delta_s\}$ in figure 2(a) is more apparent than that of $\mathrm{Re}\{\Delta_p - \Delta_s\}$ in figure 2(b). The gray level in figure 2(a) attenuates gradually with the decrease of mouse and rat IgG concentrations, indicating that the difference in concentration for both IgGs can be distinguished by $\mathrm{Im}\{\Delta_p - \Delta_s\}$ of the OI-RD.

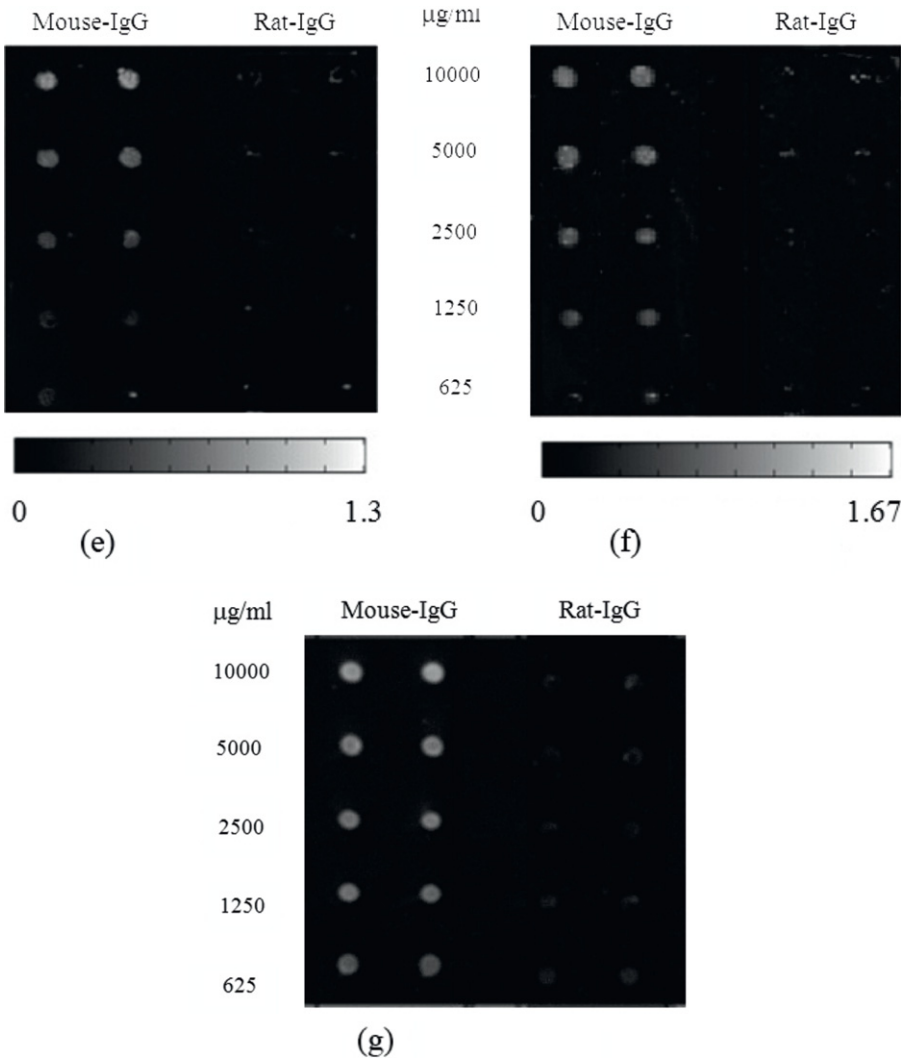

Figure 3. (Continued.)

Figures 3(a) and (b) display the 2D scan images of $\mathrm{Im}\{\Delta_p - \Delta_s\}$ and $\mathrm{Re}\{\Delta_p - \Delta_s\}$ for the aforementioned microarray after blocking respectively. It is observed from figure 3(a) that after blocking $\mathrm{Im}\{\Delta_p - \Delta_s\}$ can respond to the difference in concentrations for both mouse and rat IgG in a label-free fashion, since one uniform layer of glycine was simply added beyond the sample spot area during the blocking procedure. This leads to the change in the OI-RD intensity of the spot area relative to the unprinted background.

Figures 3(c) and (d) display the 2D scan images of $\mathrm{Im}\{\Delta_p - \Delta_s\}$ and $\mathrm{Re}\{\Delta_p - \Delta_s\}$ after the microarray was reacted with Cy5-labeled goat anti-mouse IgG. Comparing figure 3(c) with (a) and figure 3(d) with (b), it is shown that either $\mathrm{Im}\{\Delta_p - \Delta_s\}$ or $\mathrm{Re}\{\Delta_p - \Delta_s\}$ changes distinctly for mouse IgG spots while remaining unchanged for rat IgG. To reflect on the optical signals in response to antibody–antigen capture, the differential treatments were performed for both $\mathrm{Im}\{\Delta_p - \Delta_s\}$ and $\mathrm{Re}\{\Delta_p - \Delta_s\}$. Typically, figure 3(e) is the differential $\mathrm{Im}\{\Delta_p - \Delta_s\}$ obtained by subtracting figure 3(a) from figure 3(c), while figure 3(f) is the differential $\mathrm{Re}\{\Delta_p - \Delta_s\}$ obtained by subtracting figure 3(b) from figure 3(d). Meanwhile, both figures 3(e) and (f) show the evident gradient of concentrations for mouse IgG. Clearly the goat IgG has reacted specifically with the respective target since both the OI-RD signals are enhanced strongly in the printed area for mouse IgG rather than rat IgG.

Figure 3(g) shows the 2D image of the fluorescent scan after antibody–antigen capture for the same microarray in figure 3. In comparison with the images of $\mathrm{Im}\{\Delta_p - \Delta_s\}$ and $\mathrm{Re}\{\Delta_p - \Delta_s\}$ in figures 3(e) and (f), the detection results of $\mathrm{Im}\{\Delta_p - \Delta_s\}$ and $\mathrm{Re}\{\Delta_p - \Delta_s\}$ are in preferable agreement with that of the fluorescent scan. In addition, it is noteworthy that the images of the differential $\mathrm{Im}\{\Delta_p - \Delta_s\}$ and $\mathrm{Re}\{\Delta_p - \Delta_s\}$ not only show the specific binding between antibody and antigen of mouse IgG, but also suggest that the special substrate is not required for the OI-RD detection because the influences of microarray upholders can almost be eliminated by the differential treatment.

To make a better analysis and contrast, the spatially averaged intensities over all sample spots of $\mathrm{Im}\{\Delta_p - \Delta_s\}$, $\mathrm{Re}\{\Delta_p - \Delta_s\}$ and fluorescence versus the protein concentrations are plotted in figure 4. The quantitative data

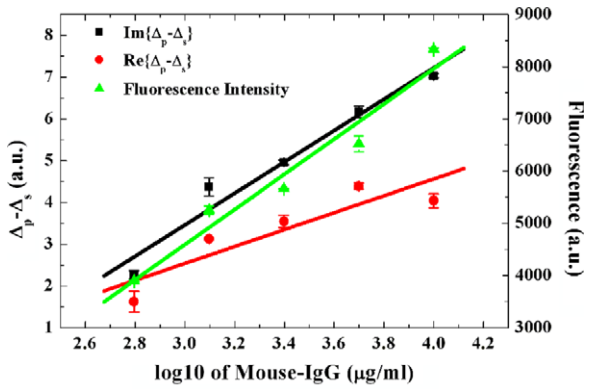

Figure 4. Protein concentrations versus the averaged intensities over each sample spot for $\mathrm{Im}\{\Delta_p - \Delta_s\}$, $\mathrm{Re}\{\Delta_p - \Delta_s\}$ and fluorescence scan, and the quantitative data of intensities are in accord with those in figures 3(e), (f) and (g), respectively.

(This figure is in colour only in the electronic version)

in figure 4 are in accord with the mouse IgG samples in figures 3(e), (f) and (g), respectively. From figure 4, it can be observed that the averaged intensities of $\mathrm{Im}\{\Delta_p - \Delta_s\}$, $\mathrm{Re}\{\Delta_p - \Delta_s\}$ and the fluorescence have a nearly linear correlation with the concentrations of mouse IgG, while the amplitude values increase with the concentration ranging from 625 to 10^4 μg ml^{-1}. In that case, the detectable mass amount by the OI-RD method is 0.688 ng with respect to 625 μg ml^{-1} because one sample spot contains about 1.1 nl solution in our experiment. On the other hand, it is difficult to detect protein in nano-grams probably as a main result of absorption based on $\mathrm{Re}\{\Delta_p - \Delta_s\}$. However, we observed the distinct $\mathrm{Re}\{\Delta_p - \Delta_s\}$ signal as shown in figure 3(f), it may derive from the absorption of Cy5 since the probe light of 632.8 nm lies in the absorption band of labeled Cy5, which needs further study and confirmation. At any rate, the experimental results prove that the OI-RD method can be applied to detect the specific binding between antibody and antigen.

4. Conclusions

By means of the OI-RD technique, the specific binding between Cy5-labeled goat anti-mouse IgG and mouse IgG with concentrations ranging from 625 to 10^4 μg ml^{-1} has been detected successfully. The experimental results demonstrate that the imaginary part of the OI-RD signal can be employed to detect the different concentrations of mouse IgG in a label-free format. Furthermore, both of the OI-RD signals coincide with the fluorescence scan for the detection of antibody–antigen capture. Especially, the differential treatment of $\mathrm{Im}\{\Delta_p - \Delta_s\}$ and $\mathrm{Re}\{\Delta_p - \Delta_s\}$ as shown in figures 3(e) and (f) can decrease or even completely remove the influences of the microarray upholder, indicating that a particular substrate does not need to be employed by the OI-RD method. Hence, it is of great advantage for the label-free and high-throughput detection of diverse biomolecular microarrays using the OI-RD method. Further investigations on detection sensitivity and high-throughput detection are being planned.

Acknowledgment

The work was supported by The National Basic Research Program in China under Project No. 2007CB935700.

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