Towards High-Throughput Label Free Detection of Biomolecular Interactions on Glass Substrates

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My summer research project as an REU student at UC Davis has mainly been an effort to create and motivate methods to achieve high-throughput screening of biomolecules from the National Cancer Institute (NCI) as possible treatments for various types of cancer. The hope of my advisor, Dr. Xiang Dong Zhu, and the rest of his group is to offer a competitive label-free drug screening technique to the market. Our microscopes use ellipsometry in a technique called oblique-incidence reflectivity difference (OI-RD) microscopy to detect biomolecular reactions *in situ* and in real time without the use of the fluorescent tagging used by all other drug screening companies and laboratories. Such a technique, if high-throughput is achieved, has powerful implications for the future of drug screening.

I. INTRODUCTION

The most effective way to study the reactive properties of a library of small molecules, macromolecules or chemical ligands is the microarray. A glass slide arrayed with 100 nm diameter spots of said molecules can be placed inside a vacuum sealing flow cell and into our OI-RD microscope. Our largest flow cell has a length of about 19mm and a width of about 36 mm; with a spot spacing of 250 nm we can array on the order of 10,000 different molecular samples onto a single slide. The library we recieved from NCI contained 8,000 small molecules, so it is feasible to react the entire library with other molecules of interest at once.

Current microarray technology is facilitated by fluorescent tagging, a highly sensitive method of labeling reactions. Fluorescent tags can be used as labels for probe molecules either extrinsically with dyes or intrinsically by genetic engineering.[1] While fluorescence offers a low background and simplified microscopy, labeling molecules ultimately has some effect on the properties of the host molecule and its interactions with other molecules. The effect of labeling is usually unknown *a priori*, and the binding affinity of a protein labeled by a fluorescent molecule such as Cy3 to a small molecule, such as those from NCI we hope to screen, may be significantly compromised.[1] A label-free method for studying proteomics would be an ideal development for more accurate characterization of molecular interactions. Ultimately a high-throughput label-free technique for screening libraries of potential small molecule cancer treatments will play a vital role in the study of biomolecular interactions.

The OI-RD microscope developed by Zhu et. al., offers just this possibility.[1] This microscope is based on the use of ellipsometry to study thin films. Ellipsometry exploits elliptically polarized light to measure the changes in magnitude and phase of the complex optical reflectivity, the Fresnel reflection coefficient, due to reactions between (in our case) immobilized molecules on a glass substrate and a liquid solution of molecules of interest. OI-RD microscopy is the most sensitive ellipsometry technique; it is polarization-modulated nulling and measures directly the fractional refectivity change between the two polarization components (s, transverse electric, and p, transverse magnetic) of the elliptically polarized laser beam.[2] The reflectivity coefficient for a bare substrate for s and p polarized light are

$$r_{s_0} = |r_{s_0}|e^{i\phi_{s_0}}$$

and

$$r_{p_0} = |r_{p_0}|e^{i\phi_{p_0}}$$

respectively. With a thin film deposited onto the surface of the substrate, the reflectivity changes to

$$r_s = |r_s|e^{i\phi_s}$$

and

$$r_p = |r_p|e^{i\phi_p}$$

. The difference in fractional reflectivity change is

$$\Delta_p - \Delta_s \equiv \frac{r_p - r_{p_0}}{r_{p_0}} - \frac{r_s - r_{s_0}}{r_{s_0}}$$

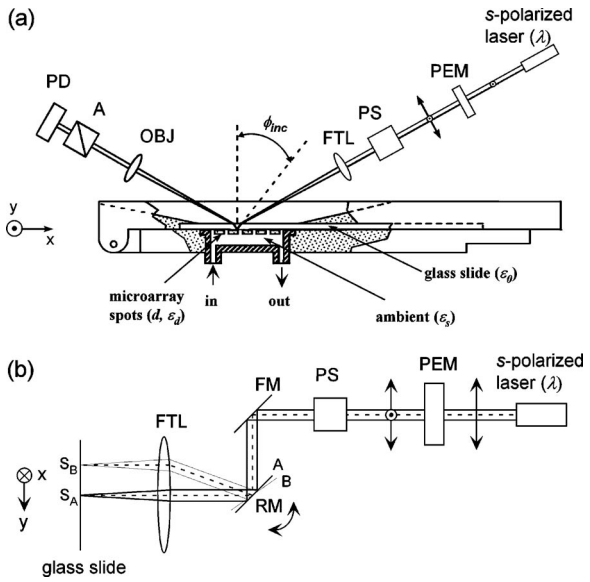

FIG. 1: Top view of a hybrid scanning OI-RD microscope with a combination of y-scan by a combination of an RM and an FTL and x-scan by translation of the sample-holding stage. The microarray-bearing surface is in contact with an aqueous solution as a part of the fluidic handling system. (b) Side view of the microscope that illustrates the y-scan. PEM: photoelastic modulator. PS: phase shifter. FM: fixed mirror. OBJ: objective lens. A: analyzer. PD: photodiode detector.

For a thin film, $\Delta_p - \Delta_s$ is small and the amplitude change of the incident light is given by

$$Re\{\Delta_p - \Delta_s\} \cong \frac{|r_p| - |r_{p_0}|}{|r_{p_0}|} - \frac{|r_s| - |r_{s_0}|}{|r_{s_0}|}$$

while the phase shift is given by

$$Im\{\Delta_p - \Delta_s\} \cong (\phi_p - \phi_{p_0}) - (\phi_s - \phi_{s_0}).$$

[3]

For a very thin film deposited onto the surface, better described as a modified surface layer, whose thickness is less than the wavelength of the incident light, the difference in fractional reflectivity change reduces to only the phase shift, the imaginary part. The binding of a protein or ligand, whose size is on the order of a nanometer and significantly smaller than the wavelength of the He-Ne laser, 632.8 nm, to the immobilized array of molecules will therefore not induce an observable magnitude shift, but will affect a phase shift which can be observed and correlated to the reaction. In relation to the incident angle of light, θ, the optical dielectric constants of the ambient, ϵ_0, film, ϵ_f, and substrate, ϵ_s, and the film thickness, d, the fractional reflectivity change is given by

$$\Delta_p - \Delta_s \cong -i \left[\frac{4\pi\epsilon_s \tan^2\theta \cos\theta}{\epsilon_0^{1/2}(\epsilon_s - \epsilon_0)(\epsilon_s/\epsilon_0 - \tan^2\theta)} \right]$$

$$\times \frac{(\epsilon_f - \epsilon_s)(\epsilon_f - \epsilon_0)}{\epsilon_d} \left(\frac{d}{\lambda} \right).$$

$\Delta_p - \Delta_s$ is maximized close to the Brewster angle of the substrate.[2] For a glass substrate, the Brewser angle is approximately $\theta_b = 56^o$ while for an air and water interface it is approximately $\theta_b = 53^o$. Our setup requires that the incident light travel through air, the glass of a flow cell, liquid in the flow cell, and to reflect off the subjacent glass slide. The maximizing angle will therefore be somewhere near that of glass and air and water. The OI-RD scanning microscope used by our laboratory is capable of varying the incident angle between 30^o and 75^o to within $\pm 0.03\mu m$. This corresponds to a spatial resolution of 5 μm.[1]

II. PROCEDURE, PROTOCOL AND PRECAUTIONS

We use the Omnigrid Microarrayer to print our slide arrays. This machine is equipped to work best with 384-well microplates as the print head is designed to hold up to 12 pins spaced according to the well spacing of the microplate. Unfortunately, the small molecule library we received from NCI came in 96-well plates. While it would have been possible to print from these plates by spacing the pins out in every other slot on the print head, the resulting array would have been difficult to print and track due to software and control limitations. So, transferring to 384-well plates is the choice method of printing as of now. Ideally we would use 32 pins to print, but this poses more complications in keeping track of the samples. Instead we used a very simple method of printing with 8 pins into 3 sets of 8 microarrays onto one slide to facilitate sample tracking. This ended up being a very time consuming process taking four 13 hour days to print the entire library and I am working to develop a method to fool the printer into printing with 32 pins to still keep track of the sample locations intuitively.

To transfer from 96 to 384 well plates, we explored the option of either transferring by hand or automation. Since there were no liquid handling systems, such as the epMotion, readily available for our use we chose to transfer by hand. We used the Matrix Equalizer 8-channel pipette whose desired feature is changeable tip spacing to facilitate movement from 96 to 384-well spacings. The transfer of one hundred 96 well plates also took longer than expected costing us two days of tiring work. By nature, manual transfers leave room for more mistakes than automated transfers and there were inevitably a couple of minor mistakes made. A drawback of automated transfers is that if there is a mistake made, we would never know and never be able to account for it. We are now looking into using an automated transfer system since fastest throughput can be achieved by using 384 well plates and we necessarily must transfer.

One particularly worrisome hurdle we faced in the transfer and printing of the small molecule library was due to the unfortunate fact that the solvent used plate the samples, Dimethyl sulfoxide (DMSO), is highly hygroscopic. A quick experiment showed that in 40% humidity, DMSO gains a volume of approximately 20% in about an hour. Subject to the less stringent requirements of fluorescence microscopy, this really wouldn't pose much of a problem, but our setup necessitates a thicker coating of molecules in a single spot. To avoid having too low a concentration per spot, we decided to double print each spot since our humidity control in the print chamber is minimal and the time the plate would be exposed is at least an hour. Of course, chances were that the plates had already been exposed and the concentration was already lower than desired. Printing twice meant that the whole print process took twice as long. It remains to be seen if this is entirely necessary and it is hoped that in the future we will be able to avoid this extra time cost.

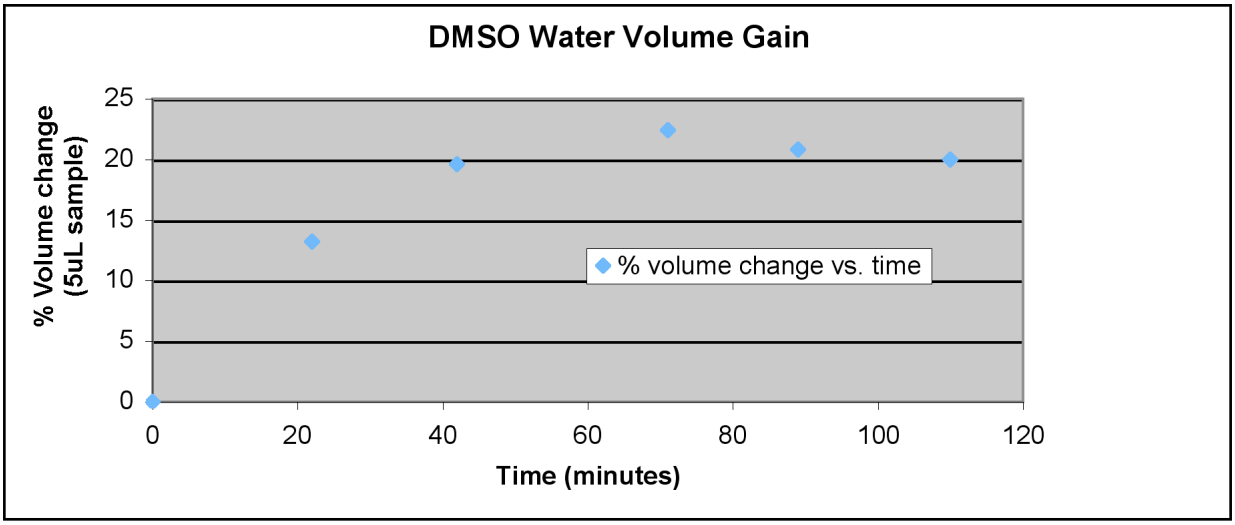

FIG. 2: DMSO hygroscopicity at 40% controlled humidity in the print chamber for a volume of 5μL

In order to create microarrays with our samples it is necessary to have some method for attaching the molecules onto a substrate, in this case, a glass slide. We attach small molecules through nucleophilic functional groups using a vapor catalyzed, isocyanate-mediated surface immobilization method.[4] Starting with amino functionalized glass slides, i.e. coated with NH_3 groups, the slides are coated with a short Fmoc-protected polyethylene glycol spacer by submersion in a solution of PEG (Fmoc-8-amino-3, 6-dioxaoctanoic acid (1 mM), PyBOP (2 mM), and DIPEA (0.5 mM) in DMF (N, N-dimethylformamide).[4] Next the slides are deprotected using piperdine and 1,6-diisocyanatohexane is coupled

to the surface by urea bond formation to create the isocyanate coating. After printing the small molecules the slides are reacted in pyridine vapor to catalyze their covalent attachment to the slide surface.[4]

III. OBJECTIVES

Ultimately we hope to screen these molecules we have printed for interactions with several proteins known to be related to cancer: MET, VEGF and 14-3-3. MET protein is also know as the Hepatocyte Growth Factor Receptor (HGFR). It is a membrane receptor that plays a vital role in embryonic development and wound healing and it is thought that stem cells exploit the normal function of MET to spread and become more persistent.[5] Vascular Endothelial Growth Factor (VEGF) and it's receptor are also candidates because VEGF is a signaling protein involved in formation of the embryonic circulatory system and the growth of blood vessels from pre-existing vasculature. VEGF has been linked to the early stages of metastasis and breast cancer. 14-3-3 is a regulatory molecule that binds to a multitude of functionally diverse signaling proteins and regulates endoplasmic reticulum localization and the surface expression of membrane proteins (i.e. the efficiency of surface transport).[6][7] So, we are looking for molecules that bind to these specific proteins in such a way to both block the ability of cancer cells to exploit the normal functions of these proteins as well as to still permit their normal function and prevent toxicity. After screening the small molecule library with our microscope for reactions with said proteins, the intent is to pass along our results to biochemists who will do further molecular studies on the reactions of these potential drugs. Achieving high-throughput for this method is our ultimate goal and has promises to be a powerful and effective process for solving such medical problems as the search for cancer treatments.

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[1] Y. Fei, J. Landry, Y. Sun, and X. Zhu, Rev. of Scientific Instruments **79** (2008).
[2] J. Landry, J. Gray, M. O'Toole, and X. Zhu, Optics Letters **31** (2006).
[3] X. Zhu, Physics Review B (2004).
[4] J. E. Bradner, O. M. McPherson, and A. N. Koehler, Nature Protocols **1** (2006).
[5] L. M. Jarvis, Chemical and Engineering News **85** (2007).
[6] B. Coblitz, M. Wu, S. Shikano, and M. Li, FEBS Letters (2006).
[7] S. Shikano, B. Coblitz, M. Wu, and M. Li, TRENDS in Cell Biology **16** (2006).