Label-Free Screening Small-Molecule Compound Libraries for Protein-Ligands Using a High-Throughput Optical Scanning Microscope

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ABSTRACT

We describe a new oblique-incidence reflectivity difference (OI-RD) scanning microscope for high-throughput screening, in microarray format on functionalized glass slides, small-molecule compound libraries for protein ligands. The microscope employs a combination of scan mirror for y-scan and single-axis translation stage for x-scan. For a printed microarray with over 10,000 features, each of 100 μm in diameter and distinct small molecule targets, we can acquire an end-point image of the microarray in 90 minutes with pixel resolution of 20 μm ×20 μm. The microscope is also capable of measuring binding kinetics of over 10,000 protein-ligand reactions simultaneously. We also describe a number of strategies for immobilizing small molecule compounds on functionalized glass slides: (1) conjugating the compounds (through a chemically inert linker) with a lysine residue so that the primary amine on the lysine serves as the anchor to epoxy-functionalized glass surface; (2) conjugating the compounds (through a linker) with a biotin residue so that the biotin serves as the anchor to streptavidin-functionalized glass surface; (3) immobilizing small molecule compounds without modification on isocyanate-functionalized glass surface through non-specific reaction of nucleophilic molecular motifs on most bioactive compounds with isocyanate groups. We present preliminary measurements of protein-small molecule binding reactions using the new microscope and the surface immobilization strategies.

Keywords: microarrays, high-throughput, label-free detection, in situ detection, oblique-incidence reflectivity difference, OI-RD, immobilization strategies

1. INTRODUCTION

Microarrays of surface-immobilized macromolecules and chemical compounds are powerful tools for highly parallel *in vitro* analysis of multiple biochemical binding events [1, 2]. They are particularly useful for proteomic and glycomic research as the number and variety of structures and functions of proteins, glycans, and their ligands are large. One of the fast-expanding microarray fields in recent years is small-molecule compound microarrays, in which small molecules are immobilized on functionalized solid surface and a solution-phase protein of interest is allowed to react with immobilized small molecule targets simultaneously in search for useful protein ligands [3, 4]. A notable challenge of small-molecule microarrays is the development of reliable, efficient methods for attaching the molecules with diverse structures and functionalities onto a functionalized solid surface. In addition, detection of protein binding to small-molecule microarrays is typically fluorescence-based such that protein probes are labeled with fluorescent tags either extrinsically or "intrinsically" through genetic engineering [5-7]. The fluorescence-based detection is widely used for its superior sensitivity and low background. However, fluorescent labeling a protein probe inevitably changes the properties of the protein in an often unspecified way. High cost, variation in labeling efficiency, and extra steps in assays are other undesirable attributes of fluorescence-based detection. It is thus sensible and also challenging to develop label-free optical detection methodology with adequate sensitivity and versatility for detection of protein binding reactions with small molecule compound microarrays at a throughput over 10,000 reactions per slide and over 100,000 reactions per day [8, 9].

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In this paper we describe (1) a recent development of a new hybrid oblique-incidence reflectivity difference (OI-RD) scanning microscope for high-throughput label-free detection of small-molecule microarrays; and (2) a number of surface chemistry platforms for immobilizing two major small-molecule compound libraries: one from one-bead-one-compound (OBOC) combinatorial synthesis with specific surface anchoring motifs for immobilization on streptavidin-functionalized or epoxy-functionalized glass surfaces; one from NCI/DTP Open Repository including natural products set with only intrinsic nucleophilic motifs for immobilization on isocyanate-functionalized glass surface.

2. INSTRUMENT

Based on measurement of oblique-incidence optical reflectivity difference (OI-RD), we developed an optical scanning microscope for label-free detection of biochemical reactions on glass-surface-supported microarrays. Here, we describe the optical arrangement and performance of a new scanning OI-RD microscope capable of detecting small molecule compounds microarrays with over 10,000 immobilized targets on a microscope glass slide. The microscope readily performs real-time as well as end-point measurements of protein-target binding reactions. Compared to imaging surface plasmon resonance (SPR) scanners [9-12], an OI-RD microscope does not rely on gold-coated substrates for detection and can have a "field of view" at least as large as 2 cm×5 cm. Compared to imaging ellipsometer based on polarizer-compensator-sample-analyzer (PCSA) scheme [13], the OI-RD microscope is inherently more sensitive to a surface-bound change (e.g., thickness, density, etc.) by at least one order of magnitude.

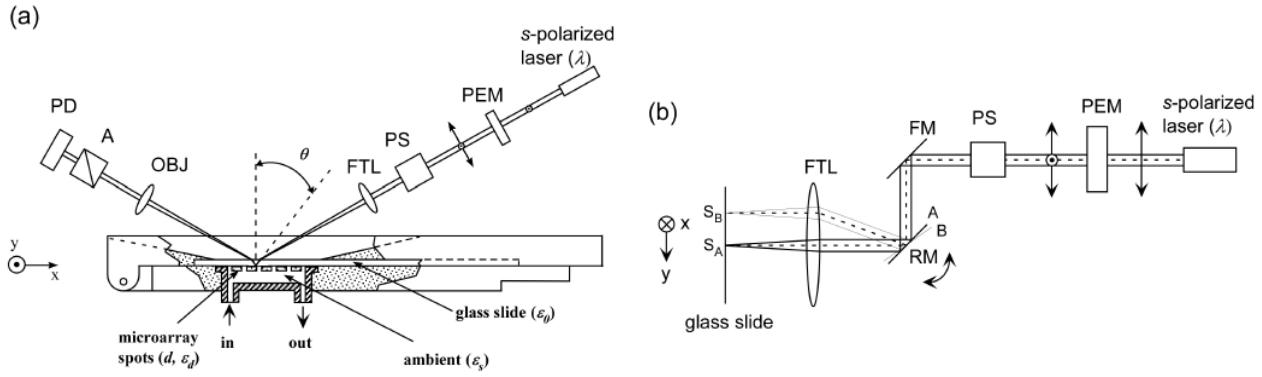

Fig.1 (a) Top view of a hybrid scanning OI-RD microscope with a combination of y-scan by a combination of a RM and a FTL and x-scan by translation of the sample-holding stage. The sample is a biomolecular microarray printed on a glass slide. The microarray-bearing surface is in contact with an aqueous solution as a part of a 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.

The optical arrangement of the scanning OI-RD microscope is sketched in Fig. 1. It is a dual-axis mechanical scanning microscope. The key difference between this scanning microscope and the previous OI-RD microscopes is that the sample-holding stage in the present microscope only moves along one axis for x-scan and the y-scan is accomplished with a galvanometer-based scan mirror. At oblique incidence a surface-bound change such as binding of protein molecules in a target-covered area alters the ratio of reflection coefficients, $r_p/r_s = \tan\psi \times \exp(i\delta)$, for p-polarized and s-polarized components of a monochromatic light. Changes in density and/or thickness of a non-absorbing layer of molecules on a transparent substrate primarily alter δ. An OI-RD microscope measures $\Delta\delta$.

As shown in Fig. 1(a), an initially *s*-polarized *continuous-wave* Nd:YAG laser beam at λ = 532 nm passes through a photo-elastic modulator (PEM) so that the resultant beam changes from being s-polarized to p-polarized at a frequency Ω. The beam then passes through a phase shifter (PS) that adds a variable phase difference Φ_{PS} between the *s*- and *p*-polarized components for nulling ellipsometric measurement. We then use an assembly of a scan mirror (RM) and an *f*-

theta lens (FTL) to focus the beam into a small spot on the microarray-bearing surface of a glass slide at incident angle $\theta = 34.66°$ from inside of the glass slide (ε_0). The microarray-bearing surface is in contact with an aqueous solution (ε_s). The reflected beam from the illuminated spot, after passing through an analyzer (A) with its transmission axis set at θ_A (45° in the present study) from p-polarization, is imaged with an objective onto a single-element photodiode (PD). The first-harmonic of the detected light intensity is given by $I(\Omega) = I_{inc}|r_p r_s|\sin(2\theta_A)\sin(\eta_{sys} + \delta + \Phi_{PS})$. η_{sys} is the phase difference between the p-polarized and s-polarized light introduced by optical components in the beam path other than the sample surface, while δ is the phase difference due to the reflection from the microarray-bearing surface. Let δ_0 be the value for the bare glass slide surface. We adjust Φ_{PS} so that on the bare surface $\eta_{sys} + \delta_0 + \Phi_{PS} = 0$ and thus $I(\Omega) = 0$. When a thin layer of molecules is subsequently added to the bare surface or when the illumination optics is moved to the region of the surface with immobilized microarray features (ε_d), $I(\Omega)$ becomes non-zero and is given by $I_{inc}|r_p r_s|\sin(2\theta_A)\sin(\delta - \delta_0)$. By independently measuring $I_{inc}|r_p r_s|\sin(2\theta_A)$, we determine $\Delta\delta = \delta - \delta_0$ directly. When the molecular layer thickness d is much less than the wavelength λ, $\Delta\delta$ is given by [14-17]

$$\Delta\delta \cong \frac{(-i)4\pi\sqrt{\varepsilon_s}\varepsilon_0}{(\varepsilon_s - \varepsilon_0)(\varepsilon_s/\varepsilon_0 - \cot^2\theta)} \frac{(\varepsilon_d - \varepsilon_0)(\varepsilon_d - \varepsilon_s)}{\varepsilon_d} \left(\frac{d}{\lambda}\right)\Theta, \quad (1)$$

where Θ is the fractional surface coverage of the molecules. From Eq. (1) it is clear that $I(\Omega)$, after the nulling step, increase linearly with d/λ [17]. An image of a biomolecular microarray on a glass slide is obtained by scanning the illumination beam in *y*-direction with the scan mirror and moving the sample-holding translation stage in *x*-direction.

To demonstrate the performance of the new scanning microscope, we printed a 10,800 spot bovine serum albumin (BSA) microarray on an epoxy-functionalized glass surface (CEL Associates, TX). A BSA molecule binds to the glass surface through reaction of its exposed amine residues with the surface epoxy groups. For printing, BSA was dissolved in 1×PBS (phosphate buffered saline) to a concentration of 7.6 μM and the solution was printed on an epoxy-coated glass slide with a contact-printing robot (OmniGrid 100, Genomic Solution, MI). The printed BSA spots have an average diameter of 100 μm and the center-center separation between neighboring BSA spots is 250 μm. After printing, the printed surface was washed with 1×PBS (*p*H=7.4) to remove excess BSA molecules. The glass slide was then assembled into a fluid chamber filled with 1×PBS. The BSA microarray was imaged *in situ* in the buffer with the new OI-RD scanning microscope. Fig. 2 shows the OI-RD image of the 10,800-spot BSA microarray. The microarray covers an area of 2 cm × 4 cm, or 8cm^2. The scan step was 20 μm in *x*-direction and 18.7 μm in *y*-direction so that the dimension of one pixel was 20 μm ×18.7 μm. The image acquisition took 90 minutes. In addition to the end-point measurement, the new scanning OI-RD microscope is also capable of following the kinetics of subsequent binding reactions on such a microarray.

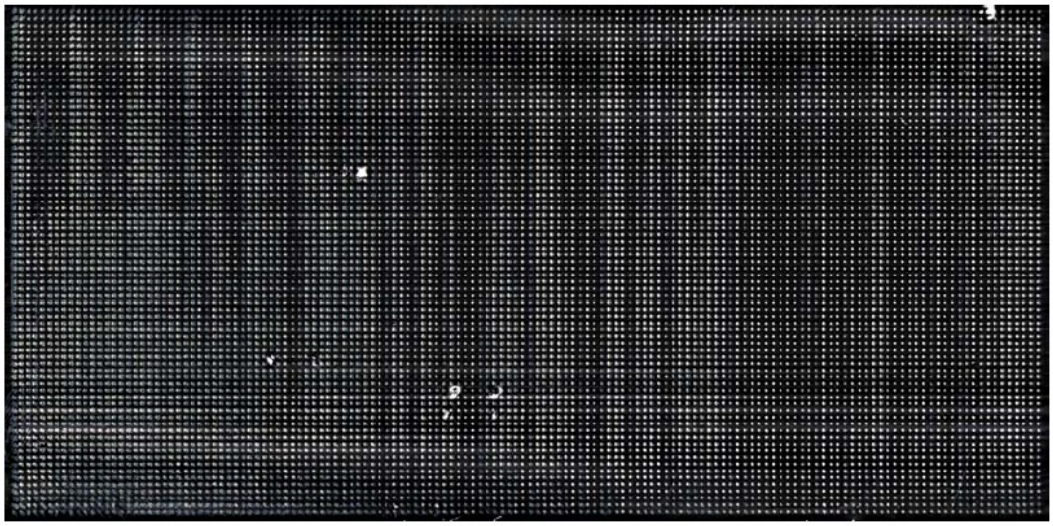

Fig. 2 OI-RD image of a 10,800-spot BSA microarray in 1×PBS acquired with the OI-RD scanning microscope as illustrated in Fig. 1. The image area ("field of view") is 2 cm × 4 cm and the pixel dimension is 20 μm × 18.7 μm.

3. STRATEGIES FOR IMMOBILIZATION OF SMALL-MOLECULE COMPOUNDS

We explored three surface chemistry schemes or strategies for immobilization of two major types of small-molecule compound libraries on suitably functionalized glass slides: (1) ~ 6,000 compounds released from microbeads synthesized by one-bead-one-compound (OBOC) combinatorial chemistry with specially conjugated biotin residues for immobilization on streptavidin-coated glass surface; (2) OBOC combinatorial compounds with specially conjugated multiple-lysine residues for immobilization on epoxy-coated glass surface; (3) ~ 8,000 mall-molecular drug sets from NCI/DTP Open Repository without additionally conjugated "molecular handles" for immobilization on isocyanate-coated glass slides.

3.1 Immobilization of small-molecule compounds synthesized via OBOC combinatorial chemistry with specifically conjugated biotin as surface anchor to streptavidin-functionalized glass surface

The OBOC combinatorial chemistry has the dual advantages of (1) rapidly producing a large library of encoded compounds built with distinct molecular structures from a handful of basic motifs, and (2) enabling addition of a common molecular motif (through a linker or otherwise) to each of synthesized compounds for surface immobilization or other purposes without unduly changing the activity of the compound [18, 19].

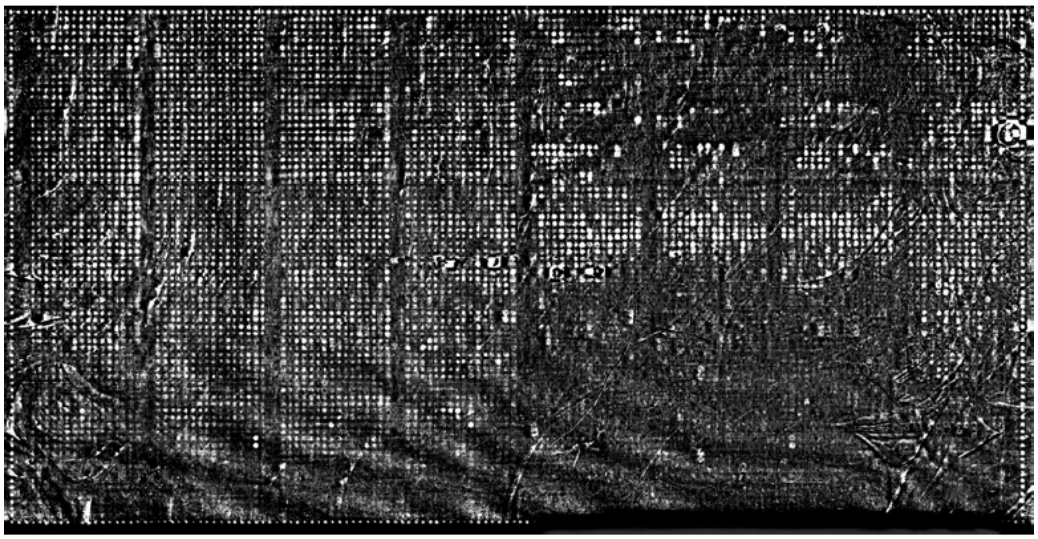

Fig. 3 The OI-RD image of a 6,000 small-molecule-compound microarray from OBOC synthesis printed on streptavidin-functionalized glass slide. The small molecule compounds were conjugated through a linker with biotin as surface immobilization anchors. The average size of printed spots is 140 μm with spot-spot separation of 250 μm.

To demonstrate the principle of detecting OBOC synthesized compounds in microarray format with our new OI-RD scanning microscope, we designed an OBOC compound library in which the surface layer of a bead displays releasable biotin-conjugated compounds and the interior of the bead displays orthogonal releasable coding tags for the compound identification [20, 21]. TentaGel resin beads (90 μm in diameter) were used as a solid support for library synthesis. Initially, TentaGel beads were topologically segregated into two layers where the outer layer with 20% of the full substitution of the beads was protected with Fmoc and the interior with 80% of the full substitution protected with Alloc. Upon Fmoc removal, a disulfide-containing linker, was tethered to the outer layer, followed by the sequential assembly of Fmoc-Lysine(Biotin)-OH, Fmoc-link-OH using Fmoc chemistry. Dde was then used to temporarily cap the N-terminus of the outer layer and allowed a CNBr-cleavable linker to be generated in the interior of a bead after Alloc deprotection using Palladium chemistry. After Fmoc deprotection, a mixture of Fmoc-Osu and Alloc-Osu (1:1) was coupled to the bead interior. Upon Dde and Fmoc deprotection, a tetra-functional scaffold was conjugated to the beads. The Alloc group was then removed by Palladium chemistry, followed by coupling with a mixture of 4-fluor-3-

nitrobenzoic acid and bromoacetic acid using HOBt and DIC. The resulting beads are split into 20 aliquots and each aliquot reacted with a primary amine in the presence of DIEA (diisopropylethylamine), respectively. The beads were mixed and reduced with tin chloride solution for 4 hours, followed by cyclization with 17 aromatic aldehyde molecules after splitting. Upon treating with acetic anhydride, the beads were Dde deprotected and 21 acids or isocyanates were acylated to the free amino group using split-mix approach. The beads were finally treated with a side-chain cleavage cocktail (TFA:water:TIS = 10:0.5:0.5) for 2.5 h to finish the desired library which contained about 7,000 distinct compounds. After synthesis, individual beads were dispensed into individual wells of polypropylene microtiter plates so that each well contained only one bead. The attached compounds were released from the beads by cutting the disulfide bond after with a TCEP solution.

These small molecule compounds conjugated with biotin through a relatively inert, flexible linker were intended to be immobilized on commercial streptavidin-functionalized glass slides (ArrayIt, CA). Biotin-streptavidin reactions are highly efficient so that forming a full layer of biotin-conjugated small molecule compounds on a streptavidin-coated glass surface only requires a compound solution concentration of 3 ~ 5 μM. Fig. 3 shows the OI-RD image of 6,000 biotin-conjugated OBOC synthesized compounds immobilized on a streptavidin-coated glass slide. Each compound was printed in duplicates that were next to each other in vertical direction. We estimated that roughly 20 picomoles of compounds were released from the outer layer of a single 90 μm-diameter bead. The outer layer has 20% of the bead volume. By adding 15 μL of TCEP in H_2O for cleavage, we made a printing solution at 1.3 μM. At such a concentration we printed twice on each spot in order to form a full monolayer of the compounds on the glass slide surface. The result displayed in Fig. 3 clearly showed that such a surface immobilization strategy using synthetically introduced biotin residue as surface anchor of an OBOC compound library is indeed high effective.

Because of high affinity of biotin while substantially lower affinity of commonly encountered molecules to streptavidin-functionalized glass surface, microarrays of biotin-conjugated small molecule compounds on such a surface need not to be blocked before subsequent protein-target reactions in many cases. There is a major drawback of streptavidin-functionalized glass slides. Being a protein-functionalized glass surface, it is relatively costly to acquire and readily subject to degradation during storage and subsequent processing. To overcome this problem, we next explored a different conjugation strategy.

3.2 Immobilization of OBOC synthesized small-molecule compounds that are conjugated with multiple lysine residues as surface anchors to epoxy-functionalized glass surface

Because superior chemical stability and low manufacturing cost, epoxy-functionalized glass slides are more appealing than streptavidin-functionalized glass slides for microarray fabrication and subsequent assays. Epoxy groups react efficiently with primary and even secondary amine residues. We have used amine-epoxy chemistry in immobilization of BSA on epoxy-functionalized glass slides. We took the advantage of OBOC synthesis by conjugating the OBOC synthesized compounds with multiple lysine residues in form of a chain or branch structure, instead of biotin. The primary amine residues on the lysine residues serve as highly efficient anchors to an epoxy-functionalized glass slide surface.

For the synthesis of a multi-lysine conjugated small molecular library using OBOC synthesis, we followed a similar procedure for the biotin-conjugated OBOC compound library as mentioned above. The difference between the two synthesis methods was how to introduce the surface anchor group motifs. In synthesis of multi-lysine conjugated compounds, a disulfide-containing linker, was first tethered to the outer layer, followed by the sequential assembly of Fmoc-Lys(Boc)-OH, Fmoc-link-OH using Fmoc chemistry. A Fmoc-Lys(Fmoc)-OH was then coupled thereafter using Fmoc Chemistry. After removal of Fmoc groups, two Boc-Lys(Boc)-OH molecules were conjugated onto amino groups. The amine residues on the lysines were subsequently used to anchor the conjugated compounds on epoxy-coated glass slide surface.

We synthesized a set of peptides which were conjugated with multi-lysine residues through a linker at the carboxyl ends. After cleavage with TCEP, we printed them on an epoxy-functionalized glass slide. The immobilization of these peptides through conjugated multi-lysine residue was nearly as efficient as that of BSA on the same surface such that forming a full monolayer of peptide targets required a printing concentration of only 10 μM. The affinities of those peptides to their respective antibodies were essentially retained after surface immobilization. Now we are in the process of synthesizing a large lysine-conjugated OBOC compound library for protein-ligand search study.

3.3 Immobilization and detection of small-molecule compound libraries from NCI/DTP Open Repository

The surface immobilization methods listed above require conjugation of a specific molecular functional group (biotin or lysine) to library compounds for orientation specific immobilization on suitably functionalized solid surfaces. The conjugation methods, while straight forward with OBOC synthesis, are not useful to a vast majority of liquid synthesized compounds that are numbered in tens of thousands or millions including extracted natural products. These compounds, some available from NCI/DTP Open Repository or NIH Small Molecule Repository or commercial vendors, have diverse structures and functional groups and thus there does not exist a one-size-fit-all conjugation chemistry yet that will allow us to add a common surface anchoring motif to all for surface immobilization into microarrays. To use the microarray platform and our OI-RD scanner to screen these compound libraries, we adopted a different surface chemistry involving non-specific reactions between an isocyanate residue with many nucleophilic residues such as (1) primary amine; (2) secondary amine; (3) indole; (4) aryl amine; (5) thiol; (6) primary alcohol; (7) secondary alcohol, (8) tertiary alcohol; (9) phenol; (10) carboxylic acid; (11) hydroxamic acid; (12) methyl; and (13) methyl ether [22,25]. As a result, over 70% of small molecule compounds react and form covalent bonds with isocyanate groups under suitable catalytic conditions. Because of relatively weaker reactions of nucleophilic groups as a whole with isocyanate group, we had to use compound solutions that were one to two orders of magnitude more concentrated in order to form a full monolayer of compounds on an isocyanate-functionalized glass surface (a small price to pay for now). Using this non-specific immobilization strategy, we successfully fabricated and detected microarrays of small-molecule drug sets from NCI/DTP Open Repository.

We adopted the protocols [23-25] described by Koehler of Broad Institute of Harvard and MIT and further optimized the procedures in our own experiments. In essence we covalently immobilized a diverse collection of small-molecule drug sets (~8,000 compounds all together) obtained from NCI on an isocyanate-coated glass slide with a vapor-catalyzed isocyanate-nucleophile reaction technique. To make isocyanate-functionalized glass slides in house, we used SuperAmine (ArrayIt, CA) functionalized slides by first coating the slides with short Fmoc-protected polyethylene glycol spacers. After deprotection using piperidine, 1,6-diisocyanatohexane was coupled to the surface by urea bond formation to provide the isocyanate-coated slides. The functionalized slides were then printed with small molecule compounds from stock solutions at concentrations of either 1 mM or 10 mM without further dilution and were subsequently exposed to pyridine vapor to catalyze the isocyanate-nucleophile reactions. Rather than quenching the printed surface immediately after the catalysis process, we stored the printed glass slide in -20°C refrigerator. Surface quenching and blocking process were commenced just before optical measurements.

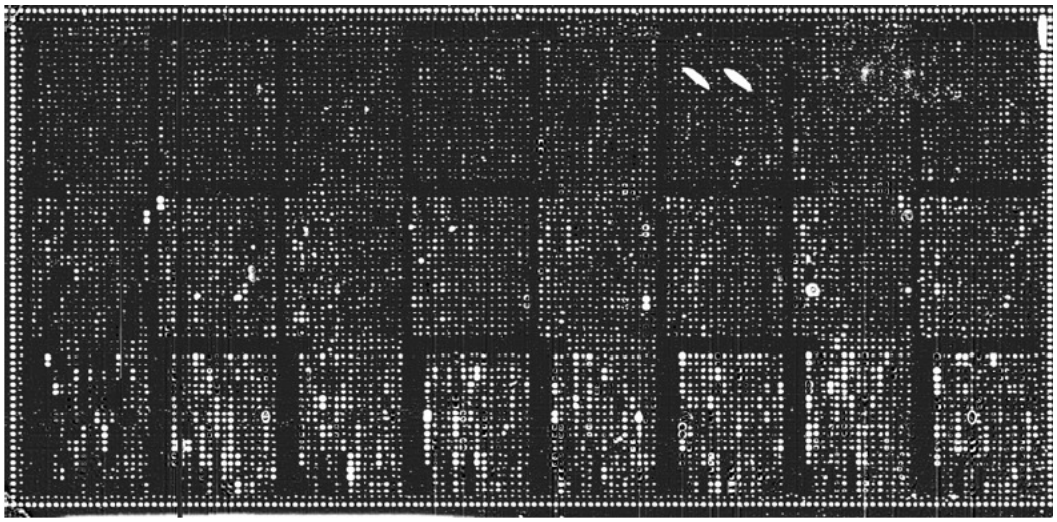

Fig. 4 OI-RD image of a small-molecule compound microarray printed on isocyanate-functionalized glass slide from stock solutions of 4,000 bioactive compounds obtained from NCI/DTP Open Repository. The compounds were immobilized through non-selective, vapor-catalyzed isocyanate-nucleophile reactions.

Fig. 4 shows the OI-RD image of 4,000 bioactive compounds obtained from NCI/DTP Open Repository printed on isocyanate-functionalized glass slide. The printing concentration is 1mM to 10mM in DMSO. Each compound was

printed in duplicate as two spots next to each other in vertical direction. The center-to-center distance is 250μm. The results show that most of the printed compounds were immobilized on the isocyanate-functionalized surface as intended. One potential disadvantage of such a non-specific surface chemistry involving an intrinsic nucleophilic residue on a compound is that the bioactivity of the compound may be compromised as a result of immobilization, particularly if the nucleophile group is the main source of the bioactivity in question. One has to be careful when interpreting the positive and negative results from such a screening. Yet the fact that over 70% of small molecule compounds from practically all sources including NCI/DTP, NIH small molecule repository, and commercial sources outweighs the disadvantage by immeasurable margin. The result in Fig. 4 clearly illustrates that the combination of isocyanate-nucleophile chemistry based surface immobilization and our high-throughput scanning optical microscope is a viable platform for rapidly screening general small molecule compound libraries for protein ligands and thus in a major way complementary to the amine-epoxy chemistry and biotin-streptavidin chemistry for screening compound libraries obtained from the OBOC combinatorial synthesis.

4. CONLUSION

We developed a new high-speed oblique-incidence reflectivity difference (OI-RD) microscope for label-free detection of over 10,000 immobilized small-molecule compounds on a single glass slide. We also explored surface chemistry strategies for immobilization of small-molecule compound libraries originated from different sources. With these platforms we have the potential to perform label-free screening of all types of small molecule compound libraries at a throughput over 10,000 compounds per glass slide. This will amount to one assay over 100,000 compounds per day on a single OI-RD scanning microscope.

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