High-throughput screening based on label-free detection of small-molecule microarrays

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ABSTRACT

Based on small-molecule microarrays (SMMs) and oblique-incidence reflectivity difference (OI-RD) scanner, we have developed a novel high-throughput drug preliminary screening platform based on label-free monitoring of direct interactions between target proteins and immobilized small molecules. The screening platform is especially attractive for screening compounds against targets of unknown function and/or structure that are not compatible with functional assay development. In this screening platform, OI-RD scanner serves as a label-free detection instrument which is able to monitor about 15,000 biomolecular interactions in a single experiment without the need to label any biomolecule. Besides, SMMs serves as a novel format for high-throughput screening by immobilization of tens of thousands of different compounds on a single phenyl-isocyanate functionalized glass slide. Based on the high-throughput screening platform, we sequentially screened five target proteins (purified target proteins or cell lysate containing target protein) in high-throughput and label-free mode. We found hits for respective target protein and the inhibition effects for some hits were confirmed by following functional assays. Compared to traditional high-throughput screening assay, the novel high-throughput screening platform has many advantages, including minimal sample consumption, minimal distortion of interactions through label-free detection, multi-target screening analysis, which has a great potential to be a complementary screening platform in the field of drug discovery.

Keywords: high-throughput screening, surface chemistry, small-molecule microarrays (SMMs), label-free technologies, oblique incidence reflectivity difference (OI-RD)

INTRODUCTION

In drug discovery, the objective of small-molecule compound screening is to find lead structures as candidates for further mechanistic and developmental studies. Drug discovery and development ultimately requires empirical assays of biomolecules against libraries containing 10^4 to 10^6 compounds. It has come to be expected that such a massive amount of screening assays should be done in a highly parallel manner. Traditional microplate-based high-throughput screening methods are mature and have been extensively applied in ligand fishing for many novel protein targets, but require expensive robotics and can suffer from artifacts from the artificial constructs and tags. Over the last decade, small molecule microarrays (SMMs) have emerged as major enabling platforms for high-throughput and cost-effective drug screening[1-3]. SMMs afford a large number of biochemical reactions through immobilization of large arrays of small molecules on a solid support so that the reaction of a protein probe with thousands of distinct molecules can be characterized simultaneously in a single experiment. In addition to high throughputs and time saving, these platforms consume far less reagents than conventional assays. Drug screening based on direct binding between ligand with protein targets make SMMs attractive for screening compounds against targets of unknown function and/or structure that are not compatible with functional assay development. These attributes make them indispensable tools of drug discovery.

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Binding of a protein target to SMMs has mostly been detected ex situ with fluorescence-based methods, by labeling either the protein with fluorescent tags or a secondary probe that recognizes the primary protein[1]. The fluorescence detection is widely used for its superior sensitivity and low background. However, fluorescent labeling has its shortcomings. High cost and variation in labeling efficiency are well known. In addition, tagging molecules of interest with fluorescent agents inevitably changes the properties of host molecules which subsequently changes innate characteristics of protein-ligand interaction and biochemistry involving the ligand or the protein or their complex[2]. Biomolecular interactions of equilibrium dissociation constants K_D in the range of μM to sub-mM are inevitably lost during post-reaction washing steps involved in ex-situ fluorescence-based detection methods[3]. Label-free detection methods, free of those problems, are developing quickly and become indispensable complementary detection methods for microarrays.

In addition to detection strategies for high-throughput screening, the success of microarray-based screening platforms hinges on efficient immobilization of a wide variety of small molecules on solid supports. A number of immobilization schemes exist for SMM fabrication[4-7], in which an isocyanate-functionalized surface is able to capture compounds containing nucleophilic residues covalently and thus have been used to successfully immobilize natural products, commercially available compound libraries, and oligonucleotides. One issue concerning the isocyanate capture method is the relatively low immobilization efficiency for some molecules due to their low reactivity with isocyanate, including small molecules containing carboxylic acid or secondary alcohol. Poor immobilization of those small molecules are prone to be missing in the following screening processes. To minimize false negative hits, it is thus important to find optimal ways to improve immobilization efficiencies for compounds with low isocyanate reactivity.

In this paper, we describe a combination of an ellipsometry-based optical scanner[8, 9] and small-molecule microarrays on phenyl-isocyanate functionalized glass surfaces as a high-throughput screening platform to screen small molecule libraries for protein ligands[10] as follows: (1) The label-free optical scanner is able to monitor about 15,000 biomolecular interactions in a single experiment with biomolecules immobilized in an area of 5.0 x 1.9 cm^2. (2) By adapting phenyl-isocyanate functionalized glass slide, we fabricated SMMs with an overall immobilization percentage of at least 73%. (3) Hits found for novel protein targets without known structures and known functions demonstrates the great potential of the platform as a complementary tool in the field of high-throughput drug screening.

METHODS AND MATERIALS

Oblique-Incidence Reflectivity Difference Scanning Microscope

Our optical sensor platform for large microarray detection is a scanning optical scanner based on polarization-modulated oblique-incidence reflectivity difference (OI-RD)[9, 11]. The arrangement of OI-RD scanner is shown in Figure 1. The He-Ne laser beam with λ = 633 nm passes through a polarizer (transmission axis is at 45° relative to p-polarization), a photoelastic modulator (modulation frequency is Ω and modulation axis is at 0° relative to p-polarization), and a phase shifter (optical axis is at 0° relative to p-polarization) . The resultant beam is focused by a f-theta lens on a microarray-containing surface at an incidence angle of 37.5°. After reflection and re-collimation, the beam passes through an analyzer (transmission axis is at 135° relative to p-polarization). The intensity of the transmitted beam is detected with a photodiode and two lock-in amplifiers. By normalizing the first harmonic signal by simultaneously measured second harmonic signal[12], the measured quantity Δδ is related to physical properties of the molecular layer such as thickness d as follows :

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

where ε_s, ε_o, and ε_d are the optical dielectric constants of the ambient, the solid substrate, and the molecular layer, respectively. θ is the incident angle on the substrate surface bearing samples. Δδ is the change of phase difference between the p- and s-polarization corresponding to presence of a thin film on substrate. OI-RD is capable of monitoring biomolecular interactions through detection of the molecular thickness d by detection of Δδ without the need to label any biomolecule.

Label-free detection of SMMs by OI-RD is achieved by raster scanning the beam across the back surface at a step size of 20 μm with a combination of a galvanometer mirror and a f-theta lens along the y-axis and by moving the microarray fluidic assembly relative to the beam along the x-axis with a linear stage. The effective printing area inside current flow cell is 5.0 ×1.9 cm^2 (x dimension and y dimension) which contains about 15,000 biomolecules with 250 μm spot spacing.

With the capability of large microarray detection, OI-RD is well suitable for high-throughput screening of SMMs in label-free mode which gets rid of many problems associated with labeling.

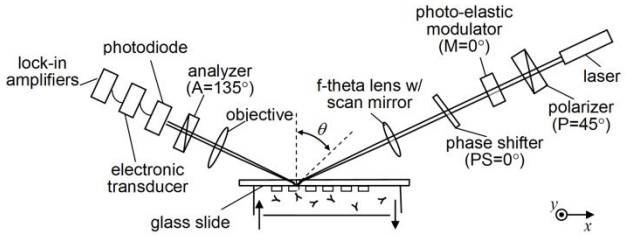

Figure 1. Schematic diagram of OI-RD system for microarray detection. Polarization optics (polarizer, photo-elastic modulator, phase shifter and analyzer) are used to analyze polarization state change of light reflection from microarray. Light scanning along y direction achieved by f-theta lens with galvanometer mirror and translation stage scanning along x direction provide spatial resolution for OI-RD system. Photodiode, electronic transducer and lock-in amplifiers are used to measure light intensity. The azimuths for polarizer, photo-elastic modulator, phase shifter and analyzer are set at P=45°, M=0°, PS=0°, and A=135°, respectively.

Fabrication of Small Molecule Microarrays (SMMs)

Figure 2 shows steps for preparing isocyanate functionalized glass slides from commercial amine functionalized glass slides10. First, PEG spacer was introduced by immersing amine functionalized glass slides into a $(PEG)_6$ solution for 10 hours with stirring. The $(PEG)_6$ solution contained 1 mM Fmoc-NH-$(PEG)_6$-$(CH2)_2$-COOH, 2 mM PyBOP and 20 mM DIPEA dissolved in DMF. Second, the protecting Fmoc group was removed by incubating the PEG-treated glass slides in a solution of piperidine (v/v 1%) in DMF for 12 hours with gentle stirring. Third, the terminal isocyanate group was added through incubation of de-protected glass slides in a solution of isocyanate in DMF for 1 hour with stirring. 1,4-phenylene diisocyanate (PPDI) solution at the concentration of 60 mM was used to add phenyl-isocyanate residues to the $(PEG)_6$ spacer. Afterwards, phenyl-isocyanate functionalized slides were rinsed with DMF and THF and dried with a stream of purified nitrogen. If not used immediately, the processed slides were stored in a –20 ºC freezer until printing with small molecules.

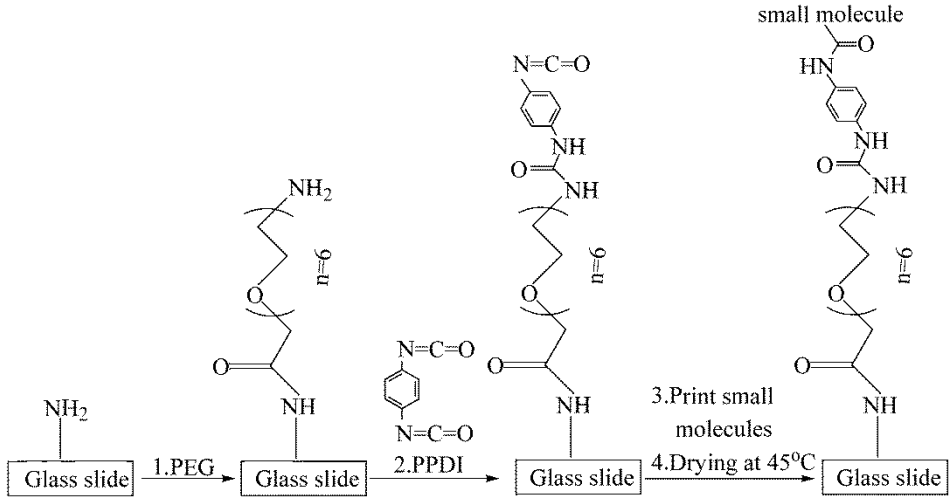

Figure 2. Steps of chemical modification of an amine functionalized glass slide to produce a phenyl-isocyanate functionalized slide and post-printing treatment to maximize small molecule immobilization.

A small molecule microarray of 3,375 bioactive compounds, including 1,053 natural compounds from Traditional Chinese Medicine (most of them from herb), 1,527 drugs approved by Food and Drug Administration (FDA) and 795 known inhibitors, was prepared as follows[10]. Each compound was dissolved in DMSO to a concentration of 10 mM and was printed in duplicate on phenyl-isocyanate functionalized slides. Biotin-BSA at a concentration of 7600 nM in 1× phosphate-buffered saline (PBS) and biotin-$(PEG)_2$-NH_2 at a concentration of 5 mM in DMSO were also printed as the

inner and the outer borders of SMMs to serve as position markers and positive controls. There were 148 columns and 64 rows in each SMM over an area of 3.7 × 1.6 cm^2. The SMM was dried at 45 ºC for 24 hours and were then stored in a -20ºC freezer until binding reactions with proteins.

Protocols for high-throughput screening based on SMMs and OI-RD scanner

For high-throughput preliminary drug screening, SMMs with 3,375 bioactive compounds was assembled into fluidic cartridge. The microarrays were (1) washed in situ with a flow of 1× PBS to remove excess unbound small molecules; (2) exposed to 7600 nM BSA in 1× PBS for 30 minutes to block unprinted isocyanate functionalized surface; (3) exposed to one target protein at a concentration of 0.01 mg/ml for 2 hours followed by washing in 1× PBS for 2 hours. (4) repeated steps (3) for all target proteins. The SMM image was acquired at each step and binding of target protein to surface immobilized small molecules was determined as bright doublets from OI-RD difference image between SMM image after and before reaction with target protein.

RESULTS AND DISCUSSION

SMMs fabricated on phenyl-isocyanate functionalized glass slides

SMMs of 3,375 bioactive compounds are prepared on phenyl-isocyanate functionalized slides followed by thermal annealing at 45°C for 24 hours. Figure 3 shows OI-RD image of printed microarray before washing and there are 2,982 compounds successfully transferred (not necessarily immobilized via nucleophile-isocyanate reaction) to the surface. The remaining 393 compounds are not transferred to the surface, due to poor wetting properties of their printing solutions on the isocyanate-functionalized surface. After the slide was washed with 1× PBS and dried, we took auto-fluorescence image and OI-RD image of the slide to identify compounds that are successfully immobilized through the nucleophile-isocyanate reaction. Figure 4 shows the combined auto-fluorescence/OI-RD image of a printed compound microarray on a phenyl-isocyanate functionalized slide. By counting both bright and dark doublets, we identified 2,165 compounds, out of 2,982 printed, that are immobilized successfully with an immobilization percentage of 73%. Considering that the combined image will miss those immobilized compounds (doublets) that (1) are not auto-fluorescent, (2) have no excess auto-fluorescent materials washed over them, and (3) are too small to be detected in our current OI-RD scanning scanner, the actual immobilization percentage is expected to be higher than 73%. Compared to hexyl-isocyanate functionalized chemistry which is adapted widely, we find that 1,746 compounds, out of 2,982 printed, are immobilized with an overall immobilization percentage of 59%. The markedly improved immobilization percentage of small molecule on phenyl-isocyanate glass slide should improve the quality and the range of SMM applications in high-throughput drug lead discovery.

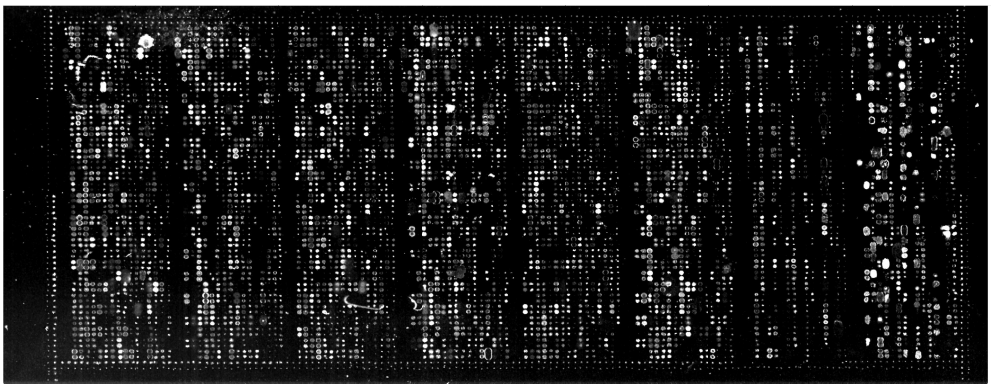

Figure 3. OI-RD image of a small molecule microarray printed from a collection of 3,375 bioactive compounds on a phenyl-isocyanate functionalized slide. The image was acquired under dry condition before washing. Solutions of 2,982 compounds were successfully transferred to the slide from the microplate during printing.

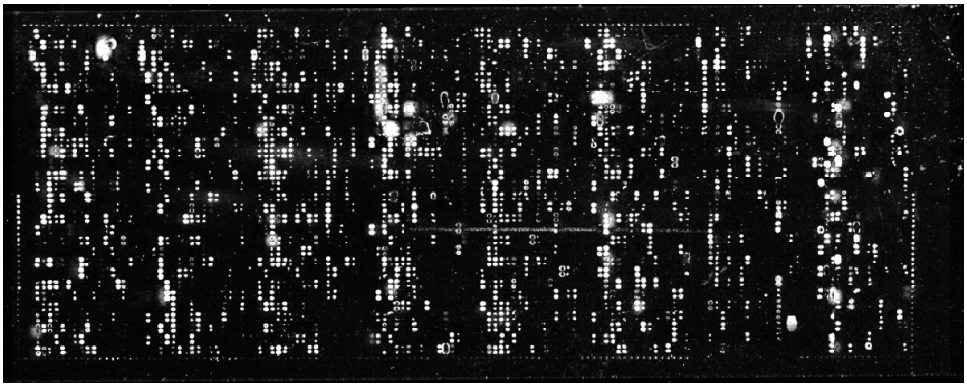

Figure 4. Combined auto-fluorescence/OI-RD image of a printed-and-washed compound microarray on a phenyl-isocyanate functionalized slide. 2,165 compounds successfully immobilized on glass slides.

High-throughput screening results based on SMMs and OI-RD scanner

Combining as-prepared SMMs on phenyl-isocyanate functionalized glass slides and label-free OI-RD scanner, we screened small molecules against several novel disease related protein targets, including (1) novel protein target of Huntington disease without known structure; (2) enzymes having difficulties in traditional high-throughput screening; (3) target protein inside cell lysate to preserve its function. Figure 5 shows one OI-RD difference image between SMM image after reaction and before reaction with strepavidin in which those bright doublets are small molecules binding to target protein. In this way, we respectively found hits for each target protein and inhibition effect of some hits were confirmed by following cell or functional assays. Our preliminary results based on combination of SMMs and OI-RD scanner demonstrates its great potential of being a complementary screening platform for drug discovery with many advantages, including minimal sample consumption, minimal distortion of interactions through label-free detection, multi-target screening analysis.

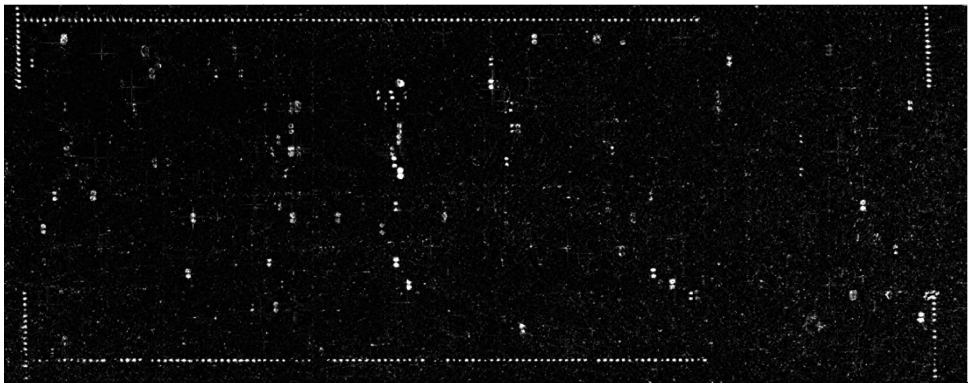

Figure 5. Changes after reaction with streptavidin in OI-RD image of small molecule microarrays with 3,375 compounds printed on phenyl-isocyanate functionalized slide and 22 compounds were identified to have reacted with streptavidin.

CONCLUSIONS

The results presented here demonstrate the feasibility and versatility of the combined SMMs and OI-RD scanner for high-throughput screening of small-molecule libraries against a variety of protein probes with different biological functions and molecular weights. The platform enables label-free, high-throughput, automated, and cost-effective screening which has great potential to be a complementary tool in drug discovery.

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