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A Robust Small-Molecule Microarray Platform for Screening Cell Lysates

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Summary

Herein we report the expanded functional group compatibility of small-molecule microarrays to include immobilization of primary alcohols, secondary alcohols, phenols, carboxylic acids, hydroxamic acids, thiols, and amines on a single slide surface. Small-molecule "diversity microarrays" containing nearly 10,000 known bioactive small molecules, natural products, and small molecules originating from several diversity-oriented syntheses were produced by using an isocyanate-mediated covalent capture strategy. Selected printed bioactive compounds were detected with antibodies against compounds of interest. The new surface of the diversity microarrays is highly compatible with approaches involving cellular lysates. This feature has enabled a robust, optimized screening methodology using cellular lysates, allowing the detection of specific interactions with a broad range of binding affinity by using epitope-tagged or chimeric fluorescent proteins without prior purification. We believe that this expanded research capability has considerable promise in biology and medicine.

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Introduction

Natural products and products of diversity-oriented synthesis (DOS) constitute a rich pool of small molecules from which specific ligands to proteins of interest may be found [1]. Small-molecule microarrays [2–11] (SMMs) enable the discovery of previously unknown protein-ligand interactions, resulting in small-molecule modulators of protein function [12, 13]. To make SMMs, stock solutions of compounds are robotically arrayed onto functionalized glass microscope slides that are incubated with proteins of interest. Microarray features representing putative interactions between proteins and small molecules are typically visualized with fluorescently labeled antibodies and a standard fluorescence slide scanner.

Several mild and selective coupling reactions have been used to capture covalently synthetic compounds onto glass surfaces and include a Michael addition [10], addition of a primary alcohol to a silyl chloride [4], diazobenzylidene-mediated capture of phenols [2], 1,3-dipolar cycloaddition [3], a Diels-Alder reaction [5], a Staudinger ligation of azides onto phosphane-modified slides [7], and capture of hydrazide-linked compounds onto epoxide-functionalized glass and vice versa [8, 9]. Most of these surface capture methods take advantage of a reactive functional group that is introduced as part of a solid-phase organic synthesis and biases the orientation of the small molecule on the surface [7, 14]. Nonselective photoinduced crosslinking has also been used to immobilize a set of ten complex natural products onto glass slides [6]. Noncovalent approaches have also been employed, such as the hybridization of peptide-nucleic acid conjugates to oligonucleotide arrays [15, 16].

Using selective approaches, our laboratories have immobilized over 50,000 products of diversity-oriented synthesis pathways via capture through a primary or secondary alcohol on chlorinated slides or through capture of phenols on diazobenzylidene-functionalized slides [2, 4, 12]. Unfortunately, the previous approaches forced us to make separate microarrays for compounds that contained either a primary or secondary alcohol and compounds containing aryl alcohols. We hoped to develop arrays that would capture all three types of alcohols on a common slide surface. Additionally, we hoped to include compounds from natural sources, not necessarily bearing primary alcohols, secondary alcohols, or phenols, alongside synthetic compounds in the microarrays. We turned to nonselective photoinduced crosslinking as a capture method and experienced mixed results. Although we successfully printed and detected several of the known ligands described by Kanoh et al. [6], our attempts to print and screen microarrays of 6336 phenol-containing fused bicycles and tetracycles [2, 17] provided unacceptable numbers of false positives as judged by secondary binding assays using surface plasmon resonance. This experience led us to pursue new capture strategies that would allow immobilization of several common functional

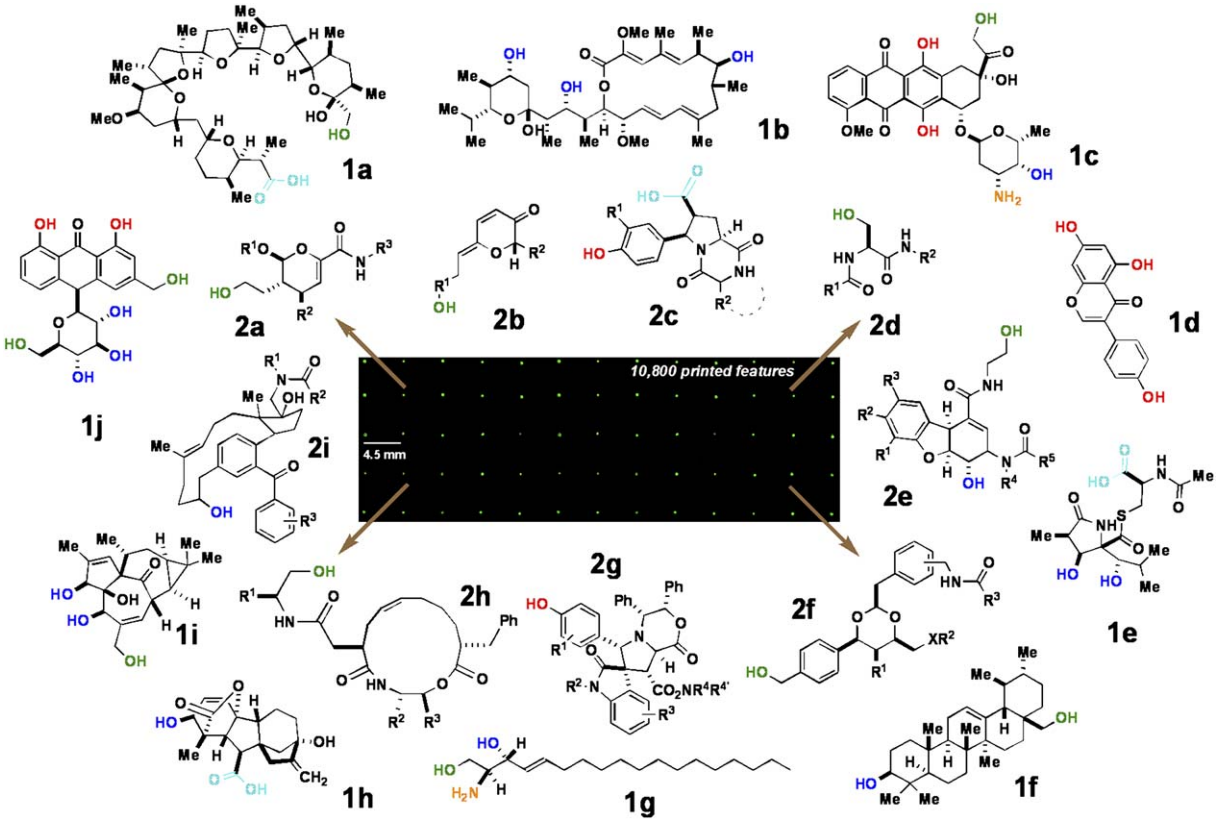

Figure 1. Schematic Design of the Diversity SMM Containing Bioactive Small Molecules and Products of Diversity-Oriented Synthesis

Reactive functional groups are colored. Representative bioactive small molecules printed in the diversity array include **1a**, nigericin; **1b**, bafilomycin A1; **1c**, doxorubicin; **1d**, genistein; **1e**, lactacystin; **1f**, uvaol; **1g**, D-*erythro*-sphingosine; **1h**, gibberellic acid; **1i**, ingenol; **1j**, aloin. Representative scaffolds for DOS small molecules printed in the diversity array include **2a**, dihydropyrancarboxamides [23]; **2b**, alkylidene-pyran-3-ones [18, 19]; **2c**, fused pyrrolidines [20]; **2d**, serine-derived peptidomimetics; **2e**, shikimic acid-derived compounds; **2f**, 1,3-dioxanes [24]; **2g**, spirooxindoles [22]; **2h**, macrocyclic lactones; **2i**, ansa-seco steroid-derived compounds [21].

groups that are present in both synthetic and natural compounds.

We have previously reported the use of SMMs to discover ligands for calmodulin (calmoduphilins) [2], the yeast transcriptional corepressor Ure2p (uretupamines) [13], and the Hap3p subunit of the yeast HAP transcription factor complex (haptamides) [12]. Each of these screens involved SMMs in which only one DOS library was contained on a given slide. More recently, we sought to prepare an SMM that contains sublibraries from various DOS synthetic routes in one array. The goal of preparing such an SMM is to allow researchers to sample the various sublibraries in one array and then prioritize screens of the full DOS libraries based on the initial screening results from the diverse subset. Here we report the use of isocyanate-functionalized glass slides to make a small-molecule "diversity microarray" containing several collections of DOS compounds coming from various solid-phase organic synthesis routes [18–24] and commercially available bioactive compounds, including natural products, on the same slide (Figure 1). Isocyanates react with a number of nucleophilic functional groups without leaving an acidic byproduct [25] and an isocyanate surface thereby increases the diversity of small molecules, from natural or synthetic sources, that can be immobilized onto a single SMM. Isocyanate glass substrates have also been prepared and used to immobilize oligonucleotides in a microarray format [26–29].

Prior strategies aimed at ligand discovery using SMMs have relied on incubation with a purified protein of interest. Potential applications of these protocols have been limited by challenges in protein biochemistry involving expression of large proteins, solubility, post-translational modification state, activity, and yield. Furthermore, without commercial availability of a protein target of interest, investigators without expertise in protein biochemistry may be limited in their capacity to screen SMMs. Here we describe the optimization of a robust, efficient SMM screening methodology which allows the detection of specific protein-small molecule interactions by using epitope-tagged target proteins directly from cell lysates without purification. We demonstrate that the new attachment chemistry is compatible with detection of known interactions between various small molecules and FKBP12 [30, 31] obtained directly from cellular lysates. Previous research reporting the detection of specific interactions with complex lysates has typically involved the addition of known, purified proteins [32] or has required incubation in solution with focused libraries of covalent probes conjugated to nucleic acids prior to spatial arraying on an

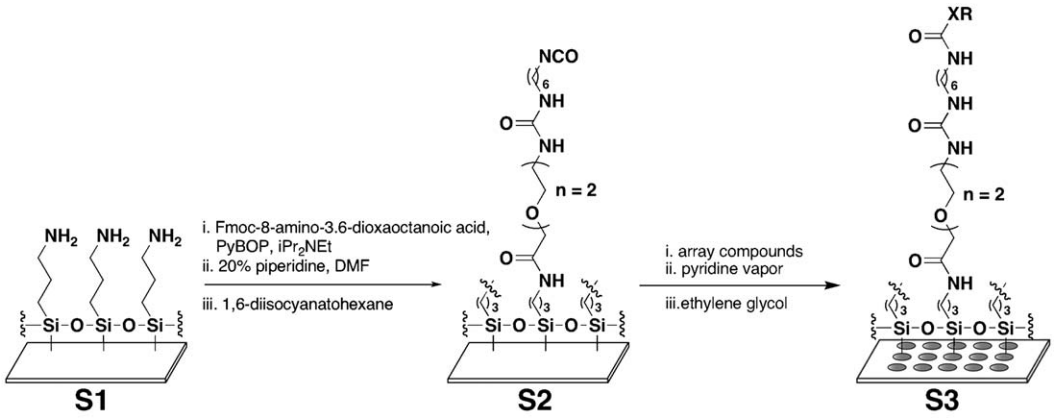

Figure 2. Vapor-Catalyzed Surface Immobilization Scheme

γ-aminopropylsilane (GAPS) slides (**S1**) are coated with a short Fmoc-protected polyethylene glycol spacer. After removal of the Fmoc group with piperidine, 1,6-diisocyanatohexane is coupled to the surface via urea bond formation to generate putative isocyanate-functionalized glass slides (**S2**). Slides printed with compound stock solutions are then placed in a dry environment and exposed to a pyridine vapor that catalyzes the covalent capture of small molecules onto the slide surface (**S3**).

oligonucleotide array [15, 16]. The ability to detect selective interactions in cellular lysates without protein purification is appealing for ligand discovery, target identification, antibody and protein specificity profiling, as well as for future applications such as signature discovery for cellular states and diagnostic tool development.

Results

Small molecules containing nucleophiles with a range of reactivities were arrayed onto a weakly electrophilic surface that reacts slowly with either the small molecules or ambient moisture and yields no potentially deleterious byproducts such as an acid. As shown in Figure 2, γ-aminopropylsilane slides (**S1**) were coated with a short polyethylene glycol (PEG) spacer and coupled to 1,6-diisocyanatohexane via a urea bond to generate putative isocyanate-functionalized glass slides (**S2**). Slides printed with compound stock solutions were then placed in a dry environment and exposed to a pyridine vapor that catalyzes the covalent capture of small molecules onto the slide surface (**S3**).

To evaluate this approach, a robotic microarrayer was used to print a series of synthetic FKBP12 ligands [33] that were derivatized so as to present a primary alcohol (**3a**, **3o**, **3p**, **3q**), secondary alcohol (**3b**), tertiary alcohol (**3c**), phenol (**3d**), methyl ether (**3e**), carboxylic acid (**3f**), hydroxamic acid (**3g**), methyl (**3h**), thiol (**3i**), primary amine (**3j**, **3n**), secondary amine (**3k**), indole (**3l**), or aryl amine (**3m**) onto the isocyanate-derivatized slides (Figures 3A and 3B). The site of modification for each FKBP12 ligand has previously been shown to be tolerant to substitution as **3** is a parent structure for chemical inducers of dimerization [34]. The ligands were printed in serial 2-fold dilutions (10 mM to 20 μM) with DMF as a solvent. The printed slides were exposed to pyridine vapor, quenched with ethylene glycol, and washed extensively with DMF, THF, and methanol. Dried slides were probed with FKBP12-GST [30, 31], followed by a Cy5-labeled anti-GST antibody, and scanned for fluorescence at 635 nm with GenePix Pro 6.0 software (Molecular Devices, Union City, CA). As shown in Figure 3, the intensity of fluorescent signals corresponding to FKBP12-GST varied according to both the functional group presented for attachment and concentration of ligand. Feature diameter was dependent on the concentration of ligand, and at higher concentrations the average diameter was 250 μm. The primary amines, aryl amine, and thiol appear to have the highest immobilization levels. Fluorescence intensities for the primary alcohols, phenol, hydroxamic acid, secondary amine, and indole are also consistent with significant immobilization. The secondary alcohol, carboxylic acid, and tertiary alcohol were immobilized in lower amounts. At 1.25 mM, a typical concentration for our compound stock solutions, trace levels of primary amides **3e** and **3h** were detected whereas the ***N,N***-substituted amide **3r** (Figure 3D) was not. The addition of polyethylene glycol spacers of varying lengths to the ligand (**3n–3q**) did not make a significant impact on the feature morphology or fluorescence intensity when probed with purified protein. Additionally, polyethylene glycol spacers of varying lengths (n = 0, 2, 4, 8, 70) were added to surface **S2** and compared (data not shown). Surfaces with shorter PEG chains (n = 2, 4, 8) were equivalent and displayed improved signal-to-noise ratios over the surface without PEG. The surface with longer PEG chains displayed the lowest fluorescence levels in the binding assay and gave inconsistent spot morphologies.

Fluorescence levels were significantly reduced when pyridine vapor was omitted from the procedure (Figure 3D). Immobilization levels were slightly enhanced when the slides were exposed to pyridine at 37ºC (data not shown). To test the sensitivity of this capture method to moisture present in the compound stock solutions used for printing, 1 mM solutions of FKBP12 ligands **3a**, **3b**, **3c**, and **3e** in 9:1 DMF:ddH_2O were arrayed in triplicate onto isocyanate-derivatized slides (Figure 3E). Fluorescence intensities were equivalent to those of compounds printed directly from DMF. Tolerance to water is an important consideration for SMM preparation because compound stock solutions in DMF and DMSO appear to take on water over time as they

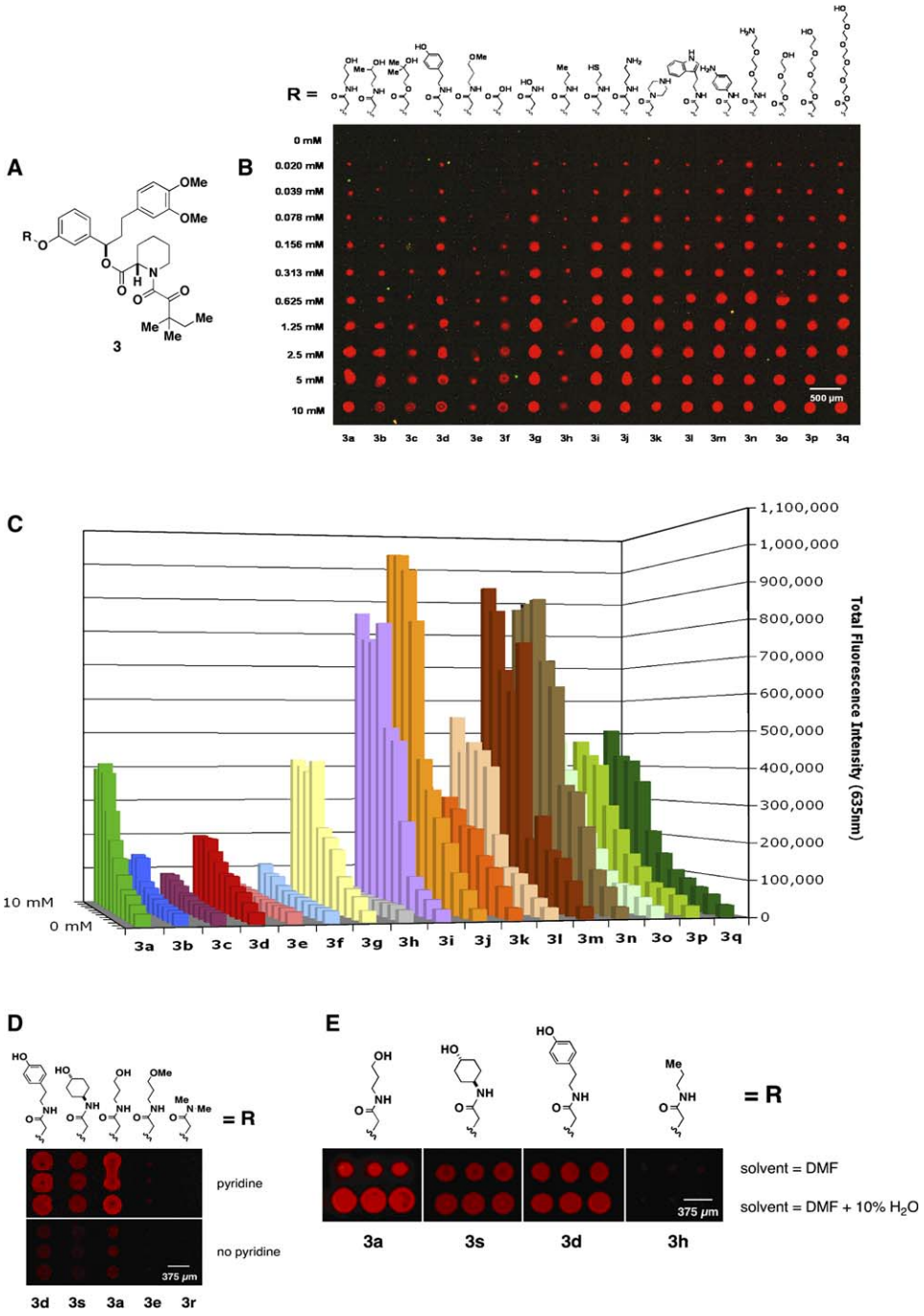

Figure 3. Comparison of Functional Group Reactivity with Isocyanate-Functionalized Glass

(A) Parent structure of AP1497 derivatives **3a–3q**.

(B) AP1497 derivative array with FKBP12 ligands **3a–3q** printed in serial 2-fold dilutions (10 mM to 20 μM) onto isocyanate-derivatized slides. The slides were exposed to pyridine vapor to catalyze the attachment of printed compounds. Washed slides were probed with FKBP12-GST followed by a Cy5-labeled anti-GST antibody. An image for a microarray scanned for fluorescence at 635 nm is shown. The functional groups presented for surface capture are shown at the top of the array.

(C) Total fluorescence intensity was computed within 300 μm spots centered on each microarray feature with GenePix Pro 6.0 microarray analysis software. The capture of small molecules is catalyzed in the presence of pyridine vapor and is tolerant of moisture in compound stock solutions.

(D) Solutions of FKBP12 ligands **3a**, **3d**, **3e**, **3r**, and **3s** (1 mM) in DMF were arrayed in triplicate onto surface **S2** and the slides were incubated either under an atmosphere of N_2 (bottom) or in the presence of pyridine vapor under an atmosphere of N_2 (top).

(E) Solutions of FKBP12 ligands **3a**, **3d**, **3h**, and **3s** (1 mM) in DMF (top row) or 9:1 DMF:ddH_2O (bottom row) were arrayed in triplicate onto isocyanate-derivatized slides.

move in and out of freezer storage [35]. Small molecules printed from DMSO were also captured by using this method with smaller feature diameters (~100–150 μm) than compounds printed from DMF (~250–300 μm) (data not shown).

To investigate the suitability of our approach for printing compounds that have not been intentionally synthesized with appendages for covalent capture, more than 300 commercially available bioactive compounds were printed onto isocyanate-functionalized slides. We screened these bioactive microarrays with rabbit primary antibodies against corticosterone, digitoxin, and 17β-estradiol, followed by a fluor-labeled goat anti-rabbit secondary antibody. The signal-to-noise ratio (SNR) was determined by calculating intensity at 635 nm and adjusting for local background for each feature on replicate arrays, and data were compared to replicate control arrays incubated with the labeled secondary antibody alone (Figure 4). Six bioactives, with SNR ratios >3.0, were found in replicate arrays to bind to the labeled polyclonal secondary antibody alone. None of the compounds were autofluorescent at 635 nm as judged by arrays probed with PBS buffer alone (data not shown). Hygromycin B, an aminoglycoside antibiotic, gave the highest adjusted SNR (mean 47.6). Three quinolone antibiotics, norfloxacin, ciprofloxacin, and pipemidic acid, displayed mean adjusted fluorescent intensities greater than 3.0 in at least one experiment. In the anti-corticosterone antibody binding profile, hydrocortisone (mean SNR 68.9), beclomethasone (63.3), and corticosterone (59.2), corticosteroids related in structure, scored as positives. Gitoxigenin (mean SNR 62.5), convallatoxin (52.7), lanatoside C (24.0), digoxin (17.8), and digitoxin (15.1), all cardioactive steroid glycosides, likewise scored as positives in replicate anti-digitoxin antibody experiments. 17β-estradiol (mean SNR 9.0), estriol (8.7), and estrone (7.3), primary estrogenic hormones varying in the number of reactive groups for capture, scored as positives in the anti-17β-estradiol binding profile. The antibody binding profiles demonstrate that small molecules with multiple nucleophilic functional groups can be printed and detected by using isocyanate-mediated capture. Additionally, these data demonstrate a facile approach for profiling the specificity of immunoglobulins for small molecules.

We aimed to expand the scope of this method to include the detection of interactions between small molecules and target proteins expressed in mammalian cells without prior purification. Toward this end, a screening protocol was developed whereby SMMs incubated with cellular lysates bearing overexpressed epitope-tagged proteins of interest are compared with control SMMs incubated with mock-transfected cellular lysates (Figure 5A). First attempts at this approach were unsuccessful due to an unfavorable interaction between the slide surface and cellular lysates prepared from a phosphate-buffered RIPA lysis buffer, yielding a uniform, high fluorescent background. By varying buffer conditions, we identified optimal signal-to-noise ratios by using an MIPP lysis buffer. These initial experiments highlight the importance of nonfluorescent detergents and buffer ionic strength, such that a balance between efficient cellular lysis and nonspecific surface interactions is achieved. Following lysis and clarification by centrifugation, cellular lysates were incubated on SMMs. Subsequently, the arrays were serially incubated with a primary anti-epitope antibody and a Cy5-conjugated secondary antibody. A brief wash with PBST and mild agitation followed each incubation. Fluorescence intensity was detected and SNR was calculated, compared, and averaged for corresponding features on replicate arrays.

We explored this approach by screening the array of AP1497 derivatives (as in Figure 3B) against HEK-293T lysates prepared from mammalian cells transiently transfected with a construct expressing FLAG-FKBP12. Optimization experiments were undertaken with a stepwise introduction of variation to identify parameters maximizing protocol robustness. Arrays were derived from the same printing series and were scanned for fluorescence by using identical laser power and photomultiplier tube gain. Experimental variables were compared by using mean SNR for ligands arrayed at a uniform, standard concentration of 1.25 mM, as depicted in Figure 5B. To determine whether the total protein concentration affects ligand detection, SMMs were incubated with lysates varying in concentration from 0.1 to 1.0 μg/μl. Maximum fluorescence intensity and SNR for each feature proved optimal at 0.3 μg/μl. Blocking incubations are commonly employed in protocols involving SMMs. Given the complex milieu of cellular lysates, we were interested in exploring whether blocking prior to sample incubation is required. Blocking with BSA was found to diminish both the maximum signal intensity and SNR when incubating SMMs with cellular lysates. Interactions between printed ligands and macromolecules may be enhanced with the introduction of a polymeric polyethylene glycol (PEG) spacer, which additionally may minimize nonspecific protein adsorption. To investigate the effect of spacer length on fluorescent detection and SNR, PEG spacer length was varied in printed AP1497 derivative SMMs. A marked decrease in the SNR was observed for each printed feature with a long ($n \sim 70$) PEG spacer compared to a substantially shorter spacer ($n = 2$). Additional optimization experiments and the detailed, optimized screening protocol for SMMs with cellular lysates are presented in Supplemental Data available with this article online.

Recognizing the high affinity of AP1497 for FKBP12 ($K_D = 8.8$ nM), we were interested in assessing the ability of this technique to identify lower affinity interactions as may be detected in screening experiments. Focused arrays of two ligands with disparate affinity for FKBP12 (Figure 6A) were printed with control bioactives. The optimized screening protocol allowed the specific identification of ligands with K_D as a high as 2.6 μM (Figure 6B) [36]. To determine whether this method would allow the detection of low-affinity interactions between small molecules and chimeric fluorescent proteins, SMMs were incubated with lysates from mammalian cells transiently transfected with a vector encoding an EGFP-FKBP12 fusion protein. Incubated slides were washed briefly with PBST and scanned for fluorescence at 488 nm. Identification of ligands with low binding affinity was observed without the requirement of primary and fluorescently labeled secondary antibodies (Figure 6C). Transient transfection of cells in tissue culture with protein expression constructs typically results in protein overexpression,

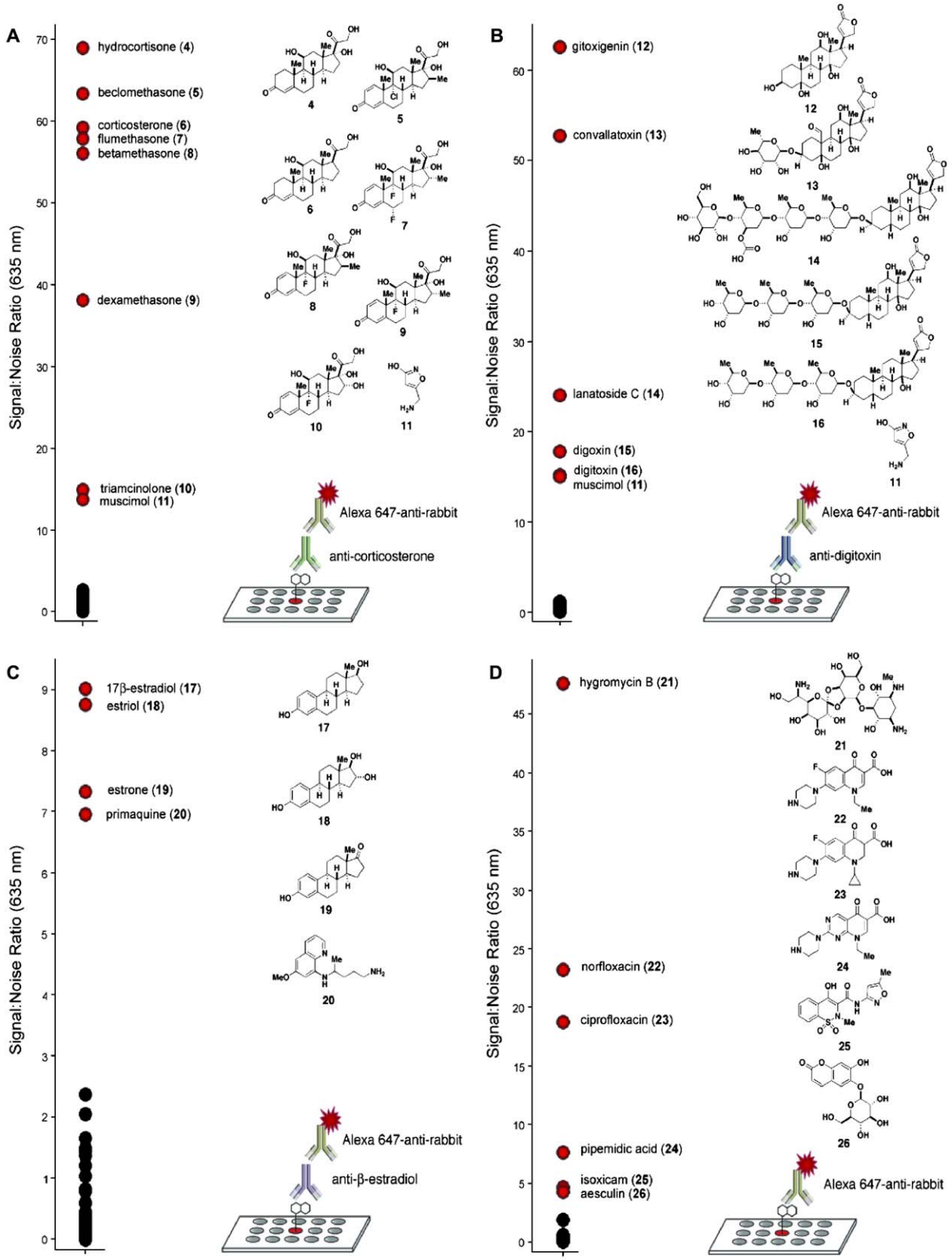

Figure 4. Detection of Selected Printed Bioactives with Antibodies

Fluorescence intensity relative to background signal for each printed bioactive is shown for binding profiles of (A) anti-corticosterone, (B) anti-digitoxin, and (C) anti-estradiol (rabbit) antibodies followed by Alexa Fluor 647 goat anti-rabbit, relative to (D) an Alexa Fluor 647 goat anti-rabbit IgG (A647 Rabbit) control. The signal-to-noise ratio at 635 nm (SNR635) is defined by (mean foreground − mean background)/(standard deviation of background). Data represent mean values of duplicate spots on an individual array confirmed by two independent experiments. All compounds with SNR635 values greater than 3.0 are labeled. Fluorescence intensity data sets for each profile are available in Supplemental Data.

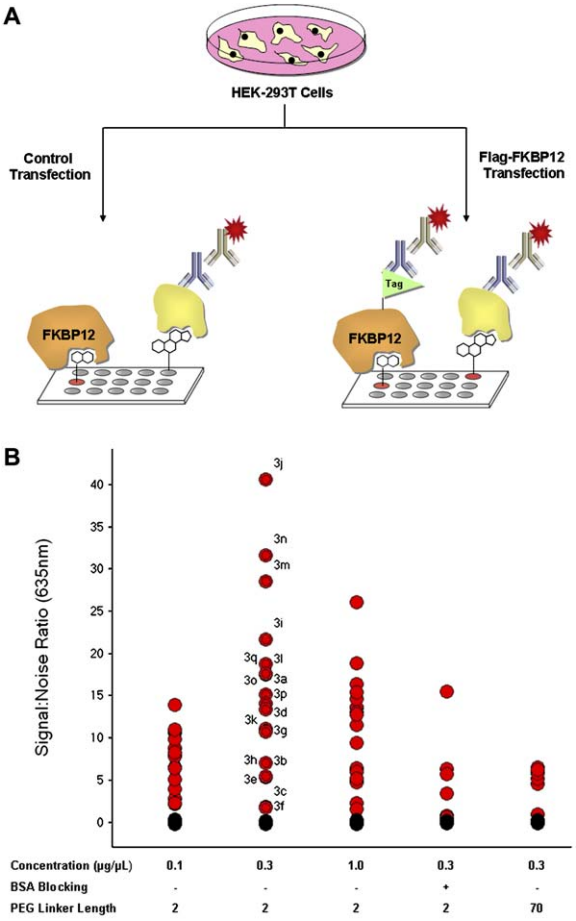

Figure 5. Screening Small-Molecule Microarrays with Cellular Lysates

(A) Schematic of the methodology. An epitope-tagged expression construct bearing a target protein of interest is introduced into a mammalian cell line by transient transfection. After 48 hr, replicate small-molecule microarrays are incubated serially with clarified lysate, primary anti-epitope antibody, and finally a fluorophore-labeled secondary antibody. A gentle, brief wash is performed in PBS following each incubation. Fluorescence intensity is computed with GenePix Pro 6.0 microarray analysis software, and intensity relative to background signal (SNR635) for each printed small molecule is compared to replicate control arrays incubated with a cellular lysate from a mock-transfected, identical cell line.

(B) Optimization of lysate screening methodology. Flag-FKBP12 overexpressed in HEK-293T cells and appropriate antibodies were selected for screening optimization experiments performed as depicted in (A) with FKBP12-ligand arrays patterned as identical triplicate subarrays with 2-fold dilutions (10 mM to 20 μM) as described in Figure 3B. Protocol conditions were serially optimized in a stepwise fashion. Data presented represent mean values (SNR635) of spots from triplicate subarrays. Data corresponding to FKBP12 derivatives **3a–3q** (red) are compared to reference, blank DMF spots (black) for experiments testing total protein concentration, the effects of blocking with bovine serum albumin (BSA), and polyethylene glycol (PEG) linker length. Complete optimization data are available in Supplemental Data.

as in the experiments above. In the context of ligand discovery, this may prove desirable; however, additional applications of SMMs such as profiling of cellular states require the detection of specific interactions with endogenously expressed proteins by using target protein-specific antibodies. To explore this possibility, SMMs were incubated with lysates from untransfected 293T cells. Subsequent incubation with a commercially available polyclonal antibody against the N-terminal region of FKBP12 and secondary fluorophore-conjugated antibody allowed the detection of specific interactions between endogenous FKBP12 and ligands with K_D as high as 2.6 μM (Figure 6D).

To investigate the robustness of our optimized lysate protocol as a screening methodology, a diverse SMM was printed containing 10,000 bioactive small molecules, natural products, and small molecules originating from diversity-oriented syntheses. The microarray also included 27 features corresponding to synthetic ligands to FKBP12 (**3–5**), and the immunosuppressant natural product rapamycin, a known ligand to FKBP12. Ten cellular lysates (five control and five Flag-FKBP12) were independently prepared and incubated with a diversity SMM. After incubation with primary and Cy5-labeled secondary antibodies, slides were scanned for fluorescence at 635 nm and feature SNR was calculated. Among five replicate SMMs with Flag-FKBP12-expressing lysate, all 27 printed ligands to FKBP12, including rapamycin and the low-affinity synthetic ligand **5**, were detected. A representative array is presented in Figure 7A.

To interrogate statistically the ability of our technique to identify ligands to a protein of interest on a diverse array, locally corrected feature intensity (SNR635) was dichotomized by a threshold intensity of 2.24, established by the maximal SNR intensity of arrayed solvent. Features with SNR intensities greater than 2.24 were classified as positives. Features from control- or Flag-FKBP12-incubated arrays were compared by using Fisher's exact test, and contingency tables were generated for 104 solvent-only features that appeared as hits in at least one experiment. At a significance level of 0.05, 24 cells were found to have a significant p value (Figure 7B). One DOS compound, 1276-M08, also scored as an assay positive. Binding was confirmed by surface plasmon resonance; however, the resynthesized, major product from the well was found to bind both GST and GST-FKBP12 by surface plasmon resonance, indicating that the molecule is likely not a selective ligand for FKBP12.

Discussion

We used a covalent capture strategy for small molecules that makes use of a well-characterized chemical reaction [25–29] and allows preparation for the first time of microarrays containing small molecules coming from both natural and synthetic sources. The isocyanate-mediated capture is applicable to compounds containing a variety of nucleophilic functional groups and does not require compounds to contain a special reactive appendage, such as an alcohol or azide [2, 4, 7], to be introduced during synthesis for covalent capture in the array. The isocyanate functionality generates no byproducts, in contrast to previous capture agents developed in our lab, including those using electropositive chlorine moieties [4]. The latter resulted in the deposition and concentration of an acidic residue in the vicinity of the small molecule, which could result in partial degradation of the small molecule and obfuscation of the screening

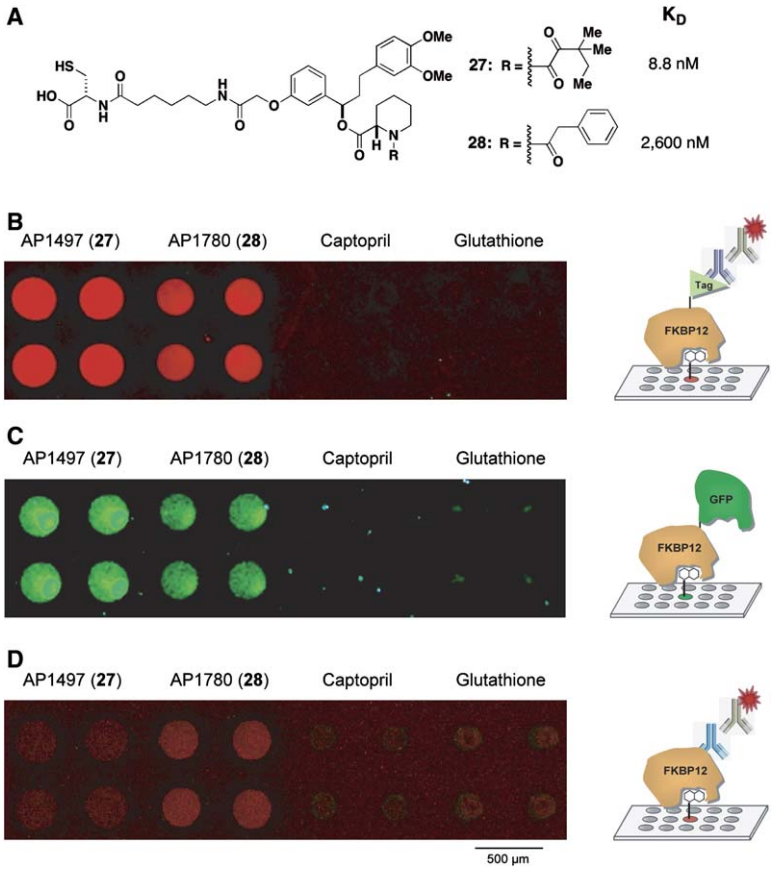

Figure 6. Detection of Binding to Ligands of Varying Affinity by Using Cellular Lysates

(A) Derivatives of AP1497 with varying affinities for FKBP12 (**27**, **28**) were obtained and printed in quadruplicate with control compounds captopril and glutathione.

(B) Arrays were incubated with clarified lysates of HEK-293T cells overexpressing Flag-FKBP12 and appropriate antibodies as depicted in Figure 5A. A false-colored, representative image of an array scanned for fluorescence at 635 nm is shown.

(C) Arrays were incubated with clarified lysates of HEK-293T cells overexpressing EGFP-FKBP12. A false-colored, demonstrative image of an array scanned for fluorescence at 488 nm is shown.

(D) Arrays were incubated with clarified lysates of untransfected HEK-293T cells and probed with a polyclonal antibody against FKBP12. A false-colored, representative image of an array scanned for fluorescence at 635 nm is shown.

results. Compounds containing multiple nucleophilic functional groups also have the potential to be displayed in varying orientations in a given spot. Multiple modes of display may allow proteins to sample different binding orientations in a given microarray feature. The isocyanate slides may, however, react with a nucleophile that is required for protein binding and may therefore lead to false negatives in screens. Due to this potential heterogeneity within printed features, we pursued surface plasmon resonance-based secondary binding assays to prioritize positives for follow-up. This approach allows us to identify rapidly candidate ligands by using the high-throughput microarray screening platform and the surface plasmon resonance platform quantitatively to characterize positives in real time.

The capture method has allowed us to produce microarrays that contain compounds derived from a variety of solid-phase syntheses alongside natural products and bioactive compounds, such as FDA-approved drugs. These arrays contain greater chemical diversity and therefore are more desirable for ligand discovery. Additionally, first screening the diversity array helps researchers select libraries for further, focused screening. Future diversity arrays will take advantage of computational methods and molecular descriptor analysis to guide decisions about which compounds to include in an effort to maximize chemical diversity.

In an effort to verify the printing of complex collections of small molecules with variable functional groups, we probed a diverse SMM with a series of antibodies with known specificities for bioactive small molecules. Structural analogs of the known target of these antibodies were also identified, indicating that large, diverse collections of printed molecules may yield insights into structure binding properties of immunoglobulins. This approach has implications for immunoglobulin profiling as has been reported previously with focused carbohydrate arrays [37]. Importantly, profiling antibody specificity among large, diverse libraries of small molecules as presented herein offers unique opportunities for rapid diagnostic, therapeutic, and neutralizing antibody discovery.

SMMs resulting from isocyanate-mediated capture are also compatible with binding screens involving total cell lysates containing endogenous or overexpressed, epitope-tagged proteins. The ability to screen directly from lysates saves substantial time and effort by avoiding protein purification, and offers the possibility of ligand discovery for proteins which have eluded comprehensive approaches at purification. Lysate screens are more biologically relevant, as many proteins of interest reside within protein complexes or require a protein partner to remain active. Proteins obtained from cellular lysates are also more likely to fold properly and possess posttranslational modifications associated with an active or desirable tertiary structure. The proteins from lysates may also serve to block the surface, thereby creating a competitive assay. The linkage of the small molecule to the surface prepared by using isocyanate capture also appears to be stable to cellular esterases and proteases under lysate screening conditions, as the slides can be stripped under denaturing conditions and reprobed

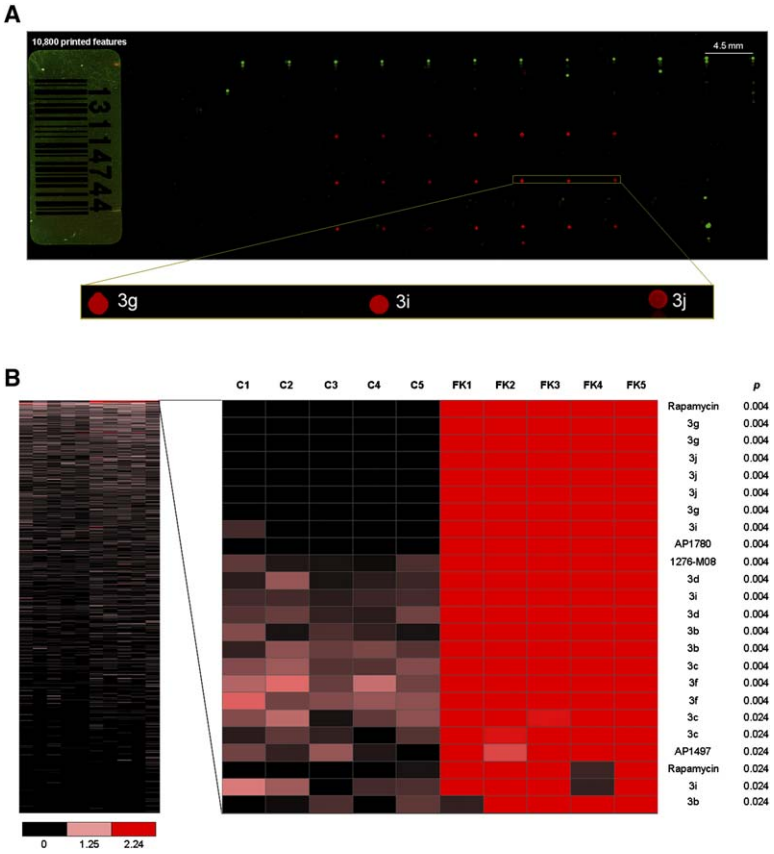

Figure 7. Analysis of Small-Molecule Microarrays Screened with Cellular Lysates

(A) An array of 10,800 features was printed with a diverse set of known bioactives, natural products, AP1497 derivatives, and compounds prepared through diversity-oriented synthesis. DMF solvent (n = 158) was included for printing to determine hit threshold intensity. Five experiments with Flag-FKBP12-overexpressing cellular lysates were compared to five incubations with control, mock-transfected lysates. Each array was subsequently incubated with an anti-Flag monoclonal antibody and a secondary Cy5-labeled anti-mouse antibody. An FKBP12-probed array scanned for fluorescence at 532 nm (green) and 635 nm (red) is shown, as well as a highlighted region demonstrating binding to AP1497 derivatives.

(B) Identification of FKBP12 binders. SNR635 profiles for five Flag-FKBP12 and five control arrays are shown. Each column is a sample on a discrete array (C, control; FK, Flag-FKBP12), and each row is a printed small molecule. The color scale indicates mean (0) and maximum (2.24) SNR635 for DMF solvent spots. Printed molecules with SNR635 above the threshold established by printed solvent and satisfying a level of significance ($p \leq 0.05$) by Fisher's exact test are presented. Complete data for each array are presented in Supplemental Data.

(data not shown). Signal-to-noise ratios in lysate experiments with isocyanate capture are improved over surfaces we have prepared that involve linkage to the surface through an ester bond. Consequently, we believe this new capability constitutes a major advance in the SMM method and should expand its use as a method to discover small-molecule partners for proteins of interest. The diversity of printed features and the compatibility of the SMM surface with this lysate screening protocol also allow profiling of complex mixtures of proteins derived from cellular lysates without prior purification. An important caveat of all applications of SMMs is the importance of antibody selection and quality. Optimization is required of all antibodies employed, especially as concerns the detection of binding events with endogenous proteins. A detailed study of lysate applications on SMMs is underway in our laboratories.

More than 1000 replicate diversity SMMs have been printed to date. Through collaborations involving several laboratories, more than 50 proteins, including single purified proteins, purified protein complexes, and proteins from clarified cell lysates, have been screened against these microarrays. Of more than 100 interactions tested, 86% retest as binders with estimated dissociation constants of 0.5–20 μM in a secondary surface plasmon resonance-based assay that involves immobilization of the target protein on a dextran-coated sensor surface and injection of the compound at varying concentrations [2]. Compounds that do not retest are typically classified as insoluble, nonspecific binders to dextran, or false positives.

In summary, we have developed a new method for preparing small-molecule microarrays that can be applied to compounds containing a range of nucleophilic functional groups, thereby increasing both the diversity and quantity of compounds, from natural or synthetic sources, that can be immobilized for microarray-based binding screens. We were able to detect and confirm the presence of selected printed small molecules, and structurally related compounds, with antibodies. Finally, we used this chemistry to prepare diversity SMMs containing nearly 10,000 small molecules and used the microarrays to demonstrate that the surface is compatible with detection of interactions using total protein from cellular lysates without any purification. Future efforts will make use of antibodies and the lysate-compatible diversity SMMs for profiling binding selectivity and changes in cell state by using small-molecule binding as a signature. We also aim to enable import of data derived from SMM experiments into the public database ChemBank.

Significance

In this manuscript, we present a new method for the preparation and screening of small-molecule microarrays. For the first time, we demonstrate the covalent capture of natural products and synthetic bioactives in significant numbers on functionalized glass microarrays. This method increases substantially the diversity, quantity, and three-dimensional display of printed small molecules. Additionally, we present

a robust, compatible method for screening small-molecule microarrays with cellular lysates from human cells. This technique enables the identification of ligands for proteins inaccessible by routine biochemical purification, greatly streamlines protein preparation, and allows screening of proteins which may require synthesis in mammalian cell lines for proper folding, complex formation, or posttranslational modification. These approaches will catalyze the efficient and widespread screening of small-molecule microarrays with proteins of interest and increase access to this technology. Additionally, they establish methods for further exploration in profiling cell states, tissues, and antibody specificity.

Experimental Procedures

Materials

Bioactive small molecules and natural products were purchased from commercial sources. DOS molecules were obtained from the Broad Chemical Biology Program. Compound **3s** was the gift of Dr. John Tallarico. Compounds **27** and **28** were obtained from Timothy Clackson of Ariad Pharmaceuticals. The Flag-FKBP12 mammalian expression construct was the gift of Dr. Paul Clemons. The EGFP-FKBP12 mammalian expression vector was constructed by using the Creator cloning system purchased from Clontech Laboratories and an FKBP12 library vector was obtained from the Harvard Institute of Proteomics. Antibodies against corticosterone, estradiol, and digitoxin were purchased from Sigma. Mouse anti-Flag monoclonal antibody was purchased from Sigma. Goat anti-FKBP12 polyclonal antibodies were purchased from Santa Cruz Biotechnology. Alexa Fluor 647 goat anti-rabbit and Alexa Fluor 647 donkey anti-goat antibodies were purchased from Invitrogen. Cy5-labeled goat anti-GST and rabbit anti-mouse antibodies were purchased from Amersham Biosciences. Slides were scanned either by using an Axon 4000B scanner at 5 μm resolution with 635 nm and 532 nm lasers or by using an Axon 4200A scanner at 5 μm resolution with 488 nm and 532 nm lasers. Arrays were analyzed with GenePix Pro 6.0 software purchased from Molecular Devices.

General Protocol for Preparation of Isocyanate Slides

Amino-functionalized glass slides, either prepared as described previously [10] or commercially available γ-aminopropylsilane (GAPS) slides (Corning), were incubated in a solution of Fmoc-8-amino-3,6-dioxaoctanoic acid (10 mM; Neosystem), PyBOP (10 mM), and *i*Pr_2NEt (20 mM) in DMF for at least 4 hr. The slides were washed in DMF to remove excess coupling solution and incubated in a solution of 10% (v/v) piperidine in DMF for 30 min (room temperature) to remove the Fmoc group from the surface. Following a rinse in DMF, the slides were activated in a solution of 10% (v/v) 1,6-diisocyanatohexane (Aldrich) in DMF for 30 min at room temperature. Three brief rinses in THF allow for complete removal of the activating solution and fast drying of the slides before placement on the robotic microarrayer platform. Depending on the length of the printing process, printed slides were allowed to dry for at least 10 min (print runs of > 2 hr) and up to 2 hr (short print runs) before they were placed onto metal racks in a glass vacuum desiccator. A three-way adaptor was attached to the desiccator, with tubing leading to a vacuum line and a round bottom flask containing approximately 1 ml of pyridine. Once the desiccator and flask were fully evacuated, the vacuum line was shut off and the catalytic pyridine vapor normalized the pressure for at least 4 hr. The slides were then immersed in a solution of ethylene glycol (1 M in DMF) and 1% (v/v) pyridine for 10 min to quench the surface. The slides were washed twice in DMF for 30 min, washed once in ethanol for 30 min, dried by centrifugation, and stored at –20ºC prior to screening. Slides were stored up to 6 months under these conditions.

Preparation of Diversity Small-Molecule Microarrays

Small molecules from the diversity set were arrayed onto isocyanate-functionalized glass slides by using an OmniGrid100 Microarrayer (Genomic Solutions) outfitted with an ArrayIt Stealth 48-pin print head and SMP3 spotting pins (TeleChem International) as described previously [2]. The microarrays contain 10,800 printed features with 48 subarrays of 15 × 15 features with 320 μm center-to-center spacing. Solutions of small molecules (~1 mM in DMF) were printed from 384-well polypropylene plates (Abgene). Twenty-eight plates containing 9152 DOS compounds [18–24], 336 bioactives, 72 control compounds, and 1192 blank wells containing DMF were printed. Forty-eight wells of a 29th plate, containing various concentrations of rhodamine derivatives (~1 mM, DMF) [10], were printed in the final dip to serve as fluorescent markers on the array that frame the subarrays. Each pin was washed three times for 5 s in acetonitrile and vacuum dried for 3 s between picking up samples from the wells in an effort to minimize carryover contamination of samples. One hundred arrays were printed in a given print run and more than 1000 copies of the diversity microarray have been printed to date. Quality control for each print run involved scanning arrays prior to screening and looking for the presence or absence of various fluor control features as well as screens to detect selected known protein-ligand interactions.

Microarray Screens with Purified FKBP12-GST

Microarrays were incubated with 300 μl of a 1 μg/ml solution of purified FKBP12-GST [30, 31] in PBST buffer (PBS [pH 7.4], 0.1% Tween-20) for 30 min at room temperature. The arrays were briefly rinsed with PBST and then washed twice in PBST (1 min for each wash) on an orbital platform shaker. Arrays were then incubated with 300 μl of a 0.5 μg/ml solution of Cy5-labeled goat anti-GST antibody in PBST for 30 min at room temperature. Probed arrays were rinsed in PBST, washed three times in PBST (2 min for each wash), and washed once in PBS (2 min). Arrays were dried by centrifugation and scanned for fluorescence at 635 nm on a GenePix 4000B microarray scanner. Control arrays were probed with either GST followed by labeled antibody or labeled antibody alone to ensure that fluorescent signals were due to binding of FKBP12 to the printed ligands. To analyze the array features containing ligands **3a–3q** (Figure 3B), total fluorescence intensity values were calculated for a set 300 μm diameter centered over each feature with GenePix Pro 6.0 software. Intensities for each ligand at varying concentrations are displayed in a graph (Figure 3C).

Small-Molecule Microarray Profiles with Antibodies against Natural Products

Microarrays printed with natural products and bioactives were incubated with various antibodies to detect specific compounds. In the first incubation step, arrays were incubated with 300 μl of one of the following: PBST buffer (control), a 1:500 solution of rabbit anti-corticosterone whole antiserum in PBST, or 1:500 solution of rabbit anti-17β-estradiol whole antiserum in PBST for 30 min at room temperature. The arrays were briefly rinsed with PBST and then washed twice in PBST. All arrays were then incubated with 300 μl of a 1:1000 solution of Alexa Fluor 647 goat anti-rabbit polyclonal secondary antibody in PBST for 30 min at room temperature. Probed arrays were rinsed in PBST, washed three times in PBST, and washed once in PBS. Arrays were dried by centrifugation and scanned for fluorescence at 635 nm. SNR was calculated for each feature with adjusted diameters.

Microarray Screens with FKBP12 from Lysates

Routine culture of HEK-293T cells was performed in DMEM supplemented with penicillin/streptomycin and 10% fetal bovine serum. Transfection of HEK-293T cells with mammalian overexpression vectors was performed by FuGENE6 lipid transfection (Roche Applied Science). Cells were harvested after 48 hr, and clarified lysates were prepared by incubation with MIPP lysis buffer (20 mM NaH_2PO_4 [pH 7.2], 1 mM Na_3VO_4, 5 mM NaF, 25 mM β-glycerophosphate, 2 mM EGTA, 2 mM EDTA, 1 mM DTT, 0.5% [v/v] Triton X-100) and centrifugation. Additional lysis buffer was added to a total protein concentration of 0.3 mg/ml, and overexpression of the target protein was verified by Western blot (data not shown). Small-molecule microarrays were serially incubated with clarified lysates, primary antibody, and an appropriate fluorophore-coupled secondary antibody. Unless otherwise specified, antibodies were diluted to 0.5 μg/ml in PBST supplemented with 1.0% bovine serum albumin. All incubations were performed for 1 hr at 4ºC. Slides were briefly washed with PBST following incubations. After a brief rinse in distilled water, slides were dried by centrifugation, scanned, and analyzed as described above. SMMs probed with lysates overexpressing EGFP-FKBP12 were incubated for 1 hr, washed briefly with

PBST, rinsed in distilled water, dried by centrifugation, and analyzed as described above. SMMs incubated with untransfected lysates for the detection of small-molecule interactions with endogenous FKBP12 were prepared and analyzed as above; however, the primary antibody was incubated at a concentration of 20 μg/ml in PBST supplemented with 1.0% bovine serum albumin.

Statistical Methods

Ten microarrays (five treatment and five control) were used to determine interactions of printed small molecules with FKBP12-containing cell lysates. Each of the microarrays contained a total of 10,800 printed features. Of the 10,800 features on each microarray, 158 features contained only solvent and were used as negative controls to establish a threshold for intensity. The maximum fluorescence intensity value (i.e., threshold) over all the solvent cells ($158 \times 10 = 1580$) was found to be 2.24. Using this threshold value to dichotomize the data, a Fisher's exact test was used to evaluate the hypothesis that the treatment cells had greater intensities than those of the control features. Contingency tables and p values were generated for 104 solvent-only features in which at least one cell demonstrated fluorescence intensity above the threshold. Calculations were performed with the exact option in SAS (Cary, NC), and no p value adjustment was made for multiple comparisons.

Supplemental Data

Supplemental Data contain a list of bioactive compounds obtained from commercial sources, spreadsheets of fluorescence intensity data for microarray screens in Figures 4, 5B, and 7, detailed information about the synthesis and analysis of ligands **3a–3r**, and SPR binding curves for 1276-M08, and are available at http://www.chembiol.com/cgi/content/full/13/5/493/DC1/. Also included is a detailed protocol for screening SMMs using cellular lysates.

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