A method for the covalent capture and screening of diverse small molecules in a microarray format

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This protocol describes a robust method for the covalent capture of small molecules with diverse reactive functional groups in microarray format, and outlines a procedure for probing small-molecule microarrays (SMMs) with proteins of interest. A vapor-catalyzed, isocyanate-mediated surface immobilization scheme is used to attach bioactive small molecules, natural products and small molecules derived from diversity-oriented synthesis pathways. Additionally, an optimized methodology for screening SMMs with purified proteins and cellular lysates is described. Finally, a suggested model for data analysis that is compatible with commercially available software is provided. These procedures enable a platform capability for discovering novel interactions with potential applications to immunoglobulin profiling, comparative analysis of cellular states and ligand discovery. With the appropriate materials and experimental setup, the printing of SMMs can be completed in 14 hours over 3 days. Screening and data analysis requires 2 days. A detailed timeline is provided.

INTRODUCTION

Among a diverse library of small molecules, the identification of specific interactions with a protein of interest is central to the discipline of ligand discovery. Typically, such interactions are identified in the context of a high-throughput screen (HTS) weighted to the functional performance of the target protein or pathway in a biochemical or cellular assay. Discovery of selective ligands in this context is frequently pursued by screening for a biophysical interaction using a primary binding assay. Downstream, secondary mechanistic studies are performed to characterize the activity of assay positives. Examples of available techniques for detecting binding interactions between small molecules and proteins include fluorescence polarization[1], isothermal calorimetry[2], fluorescence-based thermal shift[3], surface plasmon resonance[4], three-hybrid assays[5] and on-bead binding assays[6]. These approaches are limited by assay throughput, access to instrumentation or the quantity of protein substrate available for screening[7].

Small-molecule microarrays (SMMs) allow the detection of protein–ligand interactions in a parallel, high-throughput fashion and require comparably small amounts of the protein target. The initial development of this technology involved the immobilization of thiols through a Michael addition to show that detecting known interactions in a microarray format is possible[8]. Since this time, a number of notable improvements in both the covalent capture strategy and screening methodology have been contributed by many investigators[9–15]. However, most of these methods require the small molecules to contain a specific reactive functional group, such as an alcohol or azide, that needs to be introduced during synthesis for covalent capture in the microarray.

Recently, we reported the development of a novel surface to which small molecules can be attached through various nucleophilic functional groups[16]. Here, we present a detailed, step-by-step description of this method for the covalent capture of diverse collections of small molecules using the vapor-catalyzed, isocyanate-mediated technique. A schematic diagram of this approach is provided in **Figure 1**. Stock solutions of small molecules are arrayed in 384-well plate format. A protected polyethylene glycol (PEG) surface is prepared on glass microscope slides (**Fig. 2**). Following deprotection, 1,6-diisocyanatohexane is coupled to establish the reactive isocyanate surface. Small molecules are robotically printed and covalent attachment to the surface is then catalyzed by pyridine vapor. Quenched and washed slides are then stored dry for use in further experiments. The compatibility with complex natural products, products of diversity-oriented synthesis, and bioactive small molecules such as pharmaceutical agents, promises to greatly improve the quantity and structural diversity of printed small-molecule features.

This surface is experimentally compatible with assays involving clarified cellular lysates, frequently obviating the need for biochemical purification of a target. An optimized protocol for screening SMMs with purified proteins and cellular lysates is also described. Following incubation with a small volume of the protein or lysate, slides are washed and then serially incubated with a primary antibody and labeled secondary antibody. Detection of binding interactions is determined quantitatively from data collected in triplicate using standard, commercially available software developed for the analysis of printed oligonucleotide arrays. Although not described here, candidate protein–ligand interactions discovered using this protocol are typically characterized using secondary binding assays involving fluorescence-based thermal shifts and surface plasmon resonance.

There are limitations to the methods of printing and detection described in this manuscript. First, many academic environments might have limited access to chemical libraries for screening. The investment of resources and training required to establish a functional robotic microarray printing platform might also pose institutional challenges. After an initial investment of US$150,000 for equipment, the estimated cost of printing and screening SMMs is less than US$20 per array. With respect to SMM screening, many research environments have access to all reagents

and equipment necessary through microarray facilities aimed at the study of genomics.

The protocol described below involves several organic solvents and materials that require the use of appropriate safety equipment, such as safety glasses or gloves, and a properly ventilated fume hood. Notes from material safety data sheets (MSDS) are provided for selected reagents. All reactions and washes are performed in a fume hood. For more guidance on proper organic laboratory techniques, please consult ref. 17. Equipment and software are provided as examples. Alternative equipment and software can be used. The microarrays can be prepared in a microarray facility that is equipped with a properly enclosed and ventilated microarrayer as well as a neighboring fume hood. The SMMs can be screened and scanned at any standard microarray facility. In **Table 1**, we have suggested printing several commercially available dyes and small molecules, including immunosuppressant natural products and known ligands to the protein FKBP12, as test cases[18]. Applying the present protocol to these ligand–protein pairs will be of help in becoming familiar with the procedure described below.

The SMM printing and screening methodologies described below provide a blueprint for the construction of a portable, robust, parallel platform for the discovery of novel protein–ligand interactions. Prior discoveries of small molecules targeting yeast transcription factors suggest that future applications to gene regulatory elements mediating disease phenotypes, such as neoplastic transformation, will enable the identification of tool compounds and leads for further pharmaceutical development. Compatibility of the slide surface with cellular lysates creates an additional opportunity to profile cellular states or complex mixtures such as serum immunoglobulins.

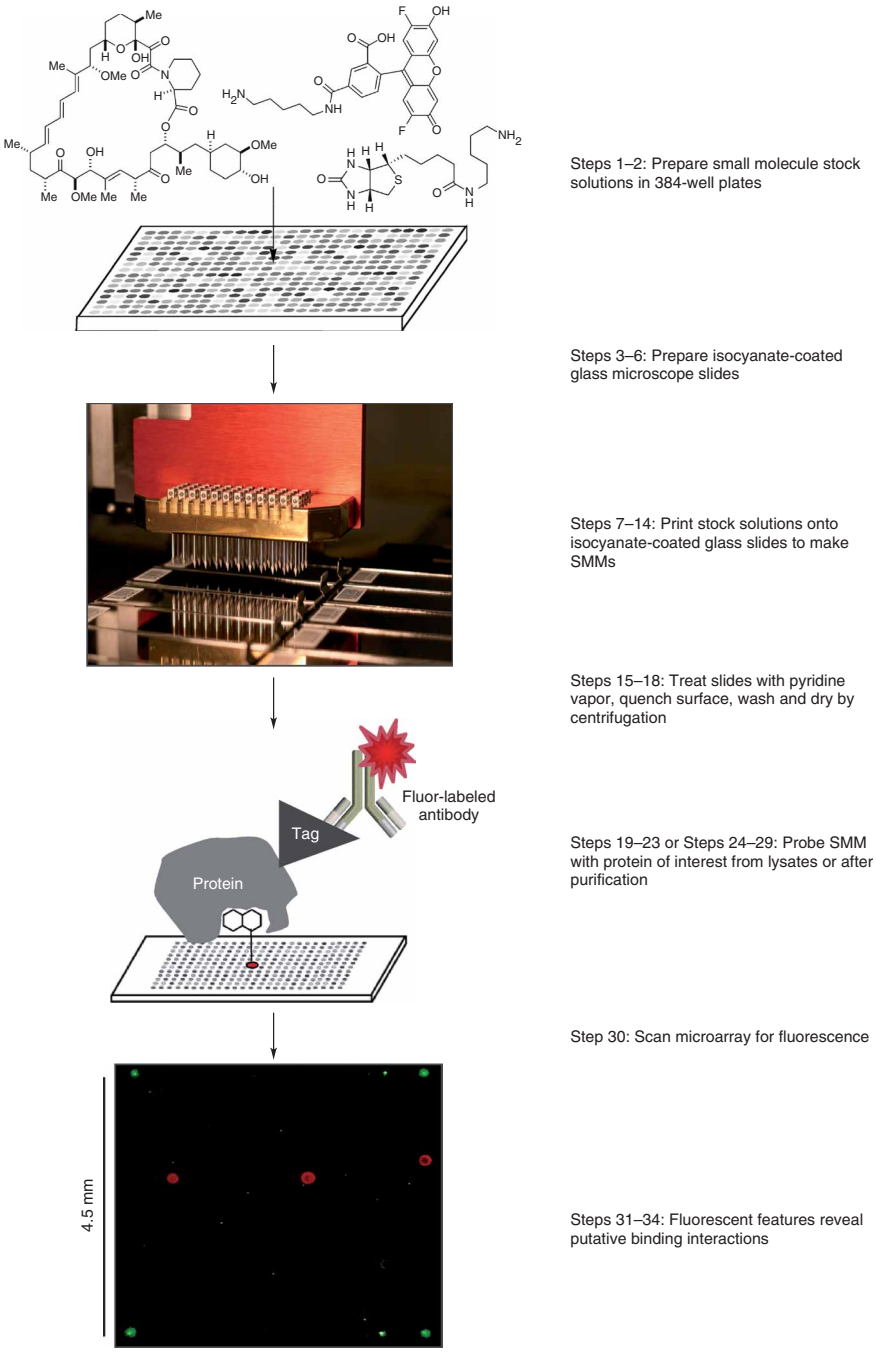

Figure 1 | A flow diagram of the small-molecule microarray (SMM) fabrication and screening process.

MATERIALS

REAGENTS

- Corning GAPS II coated glass slides (Fisher, cat. no. 07-200-006)
- Fmoc-8-amino-3,6-dioxaoctanoic acid (NeoMPS, FA03202) ▲ CRITICAL Polyethylene glycol spacers of varying lengths (n = 2–10 ethylene glycol units) have been successfully used with this protocol. Spacers of longer length (n > 30) provide lower fluorescence intensity values and inconsistent spot morphologies.
- (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluoro phosphate, PyBOP (Novabiochem, cat. no. 01-62-0016) ! CAUTION Irritant.
- Piperidine, redistilled (Sigma-Aldrich, cat. no. 411027) ! CAUTION Flammable and toxic by inhalation.
- 1,6-diisocyanatohexane (Aldrich, cat. no. D124702) ! CAUTION Toxic by inhalation and through contact with skin.
- Pyridine (Aldrich, cat. no. 270970) ! CAUTION Flammable and toxic by inhalation and through contact with skin.
- Ethylene glycol (Acros Organics, cat. no. 295530010)
- N,N-dimethylformamide (DMF) (Fisher Chemical, cat. no. D131-4) ! CAUTION Toxic by inhalation and through contact with skin.
- N,N-diisopropylethylamine (DIPEA) (Sigma-Aldrich, cat. no. 550043) ! CAUTION Flammable and corrosive.
- Dimethyl sulfoxide (DMSO) (Acros Organics, cat. no. 414880010)
- Acetonitrile (Fisher Chemical, cat. no. A998-4) ! CAUTION Flammable and toxic by inhalation and through contact with skin.

- Tetrahydrofuran (THF), stabilized (Acros Organics, cat. no. 164240025) ! CAUTION Flammable and can form explosive peroxides.
- Texas Red cadaverine (Invitrogen, cat. no. T-2425)
- Oregon Green 488 cadaverine (Invitrogen, cat. no. O-10465)
- Alexa Fluor 647 cadaverine (Invitrogen, cat. no. A-30679)
- Rapamycin (LC Laboratories, cat. no. R-5000)
- FK506 (LC Laboratories, cat. no. F-4900)
- AP1497 was prepared as described in ref. 18
- FKBP12-6xHis was prepared as described in ref. 8
- Alexa Fluor 647 conjugate anti-5xHis antibody (Qiagen, cat. no. 35370)
- Cy5 mono-reactive antibody labeling dye pack (GE Healthcare, cat. no. PA25001)
- 293T Cells (ATCC, cat. no. CRL-11268)
- Lipofectamine 2000 transfection reagent (Invitrogen, cat. no. 11668-019)
- Flag-FKBP12 mammalian overexpression construct as described in ref. 16
- Anti-FLAG M5 monoclonal mouse antibody (Sigma, cat. no. F4042)
- Anti-mouse goat secondary antibody, Alexa Fluor 647 conjugate (Invitrogen, cat. no. A-21237)
- Anti-rabbit goat secondary antibody, Alexa Fluor 647 conjugate (Invitrogen, cat. no. A-21246)
- Biotin (Sigma, cat. no. B4501)
- Biotin-PEG amine (Sigma, cat. no. B9931)
- Biotin cadaverine (Invitrogen, cat. no. A-1594)
- Streptavidin, Alexa Fluor 647 conjugate (Invitrogen, cat. no. S-32357)
- Digoxin (Sigma, cat. no. D6003)
- Anti-digoxin mouse monoclonal antibody, clone DI-22, ascites fluid (Sigma, cat. no. D8156)
- Corticosterone (Fluka, cat. no. 27840)
- Anti-corticosterone rabbit antibody, whole antiserum (Sigma, cat. no. C8784)
- Protease inhibitor cocktail tablets (Roche, cat. no. 11836170001)
- Tris-buffered saline (TBS; 0.025 M Tris-HCl, 0.137 M NaCl, 0.003 M KCl, pH 7.4)
- Tris-buffered saline with Tween-20 (TBST; 0.025 M Tris-HCl, 0.137 M NaCl, 0.003 M KCl, pH 7.4, 0.01% (vol/vol) Tween-20)
- Phosphate-buffered saline (PBS; 0.012 M NaH_2PO_4, 0.137 M NaCl, 0.003 M KCl, pH 7.4)
- Phosphate-buffered saline with Tween-20 (PBST; 0.012 M NaH_2PO_4, 0.137 M NaCl, 0.003 M KCl, pH 7.4, 0.01% (vol/vol) Tween-20)
- MIPP lysis and incubation buffer (commonly known as multiple inositol polyphosphatase buffer; 0.02 M NaH_2PO_4, 0.001 M Na_3VO_4, 0.005 M NaF, 0.025 M β-glycerophosphate, 0.002 M EGTA, 0.001 M DTT, 0.5% (vol/vol) Triton X-100, pH 7.2) ▲ CRITICAL Use of RIPA lysis and extraction buffer (commonly known as radioimmunoprecipitation assay buffer; 0.025 M Tris-HCl, 0.15 M NaCl, 1% (vol/vol) NP-40, 1% (vol/vol) sodium deoxycholate, 0.1% (vol/vol) SDS, pH 7.6) results in the formation of an autofluorescent film on the slide surface that significantly decreases the signal-to-noise ratio in the assay and should be avoided.

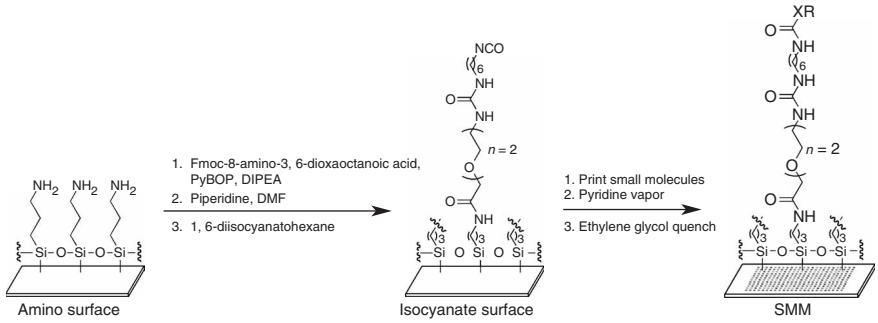

Figure 2 | Scheme for isocyanate-mediated immobilization of small molecules. Gamma aminopropyl silane (GAPS) slides are coated with a short Fmoc-protected polyethylene glycol spacer. After deprotection using piperidine, 1,6-diisocyanatohexane is coupled to the surface by urea bond formation to provide the isocyanate-coated slides used during the microarraying process. Slides printed with small-molecule stock solutions are exposed to pyridine vapor in order to catalyze the covalent attachment of molecules to the small-molecule microarray (SMM) surface. DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate.

EQUIPMENT

- OmniGrid 100 Microarrayer (Genomic Solutions)
- 946 Printhead (Telechem International, cat. no. 946PH48)
- 946 Micro spotting pins (Telechem International, cat. no. 946MP3)
- 384-well polypropylene natural microarray plates, cylindrical wells (Abgene, cat. no. AB-1055)
- Thermal peelable plate seals (Velocity11, cat. no. 06643001
- Desiccator dry storage box, acrylic (VWR, cat. no. 24987-053)
- Low-particle nitrile gloves (VWR, cat. no. 40101-222)▲ CRITICAL Use of some powdered gloves can result in autofluorescent residue on the microarrays.
- Bibulous paper (Fisher Scientific, cat. no. 11-998)
- Stainless steel 50-slide racks (Wheaton Scientific, cat. no. 900404)
- Large glass trough with stainless steel lid, 500 ml (Raymond A Lamb, cat. no. E102-6)
- Three-way glass vacuum valve with o-ring tip (Aldrich, cat. no. Z271330)
- Tygon R-3603 vacuum tubing
- Glass vacuum desiccator (Aldrich, cat. no. Z114340)
- 4-well rectangular polystyrene dishes (Nunc, cat. no. 267061)
- Square Petri dishes, 100 × 100 × 15 mm (Nunc, cat. no. 4021)
- Hybrislip hybridization covers, 60 × 22 mm (Invitrogen, cat. no. H-18202)
- 2-slide centrifuge for microarray drying (Sunergia Medical, cat. no. MSC-T)
- GenePix 4200A 4-laser slide scanner (Molecular Devices)
- GenePix Pro 6.0 software (Molecular Devices)

REAGENT SETUP

Small molecules Several small molecules that contain isocyanate-reactive functional groups are suggested as test molecules to evaluate the method (**Table 1**). Ordering information for the compounds is provided in the Reagents section. The small molecules should be diluted in DMSO to prepare 10 mM stock solutions for printing as described in Step 1.

TABLE 1 | Fluors and suggested protein–small molecule pairs for testing the protocol.

Small molecule	Protein	Screening concentration	K_D	Detection
Cy5	–	–	–	Fluorescent dye
Oregon Green	–	–	–	Fluorescent dye
Texas Red	–	–	–	Fluorescent dye
Biotin Derivatives	Streptavidin, Alexa Fluor 647	0.5 μg ml^{-1}	10^{-15} M	Fluor-labeled protein
AP1497	FKBP12-6xHis	1 μg ml^{-1}	18 nM	1:1,000 Fluor-labeled anti-5xHis antibody
FK506	FKBP12-6xHis	1 μg ml^{-1}	3 nM	1:1,000 Fluor-labeled anti-5xHis antibody
Rapamycin	FKBP12-6xHis	1 μg ml^{-1}	0.5 nM	1:1,000 Fluor-labeled anti-5xHis antibody
Corticosterone	Anti-corticosterone antibody	1:500 antiserum	Nd	1:1,000 Fluor-labeled anti-rabbit secondary antibody
Digoxin	Anti-digoxin antibody	1:500 ascites fluid	Nd	1:1,000 Fluor-labeled anti-mouse secondary antibody

K_D, dissociation constant; Nd, not determined.

Proteins The suggested test molecules can be detected with a known protein or antibody partner (**Table 1**). Printed biotin derivatives can be detected using a commercially available streptavidin-fluor conjugate as described in Step 21 (method A) and Step 22 (method A). Corticosterone and digoxin can be detected using commercial antibodies against the compounds followed by labeled secondary antibodies as described in Step 21 (method A) and Step 22 (method B). AP1497, FK506, and rapamycin can be detected by incubation with epitope-tagged FKBP12 and a labeled antibody directed against the epitope tag as described in Step 21 (method A) and Step 22 (method B). Finally, a protocol for detecting this interaction using epitope-tagged FKBP12 from cell lysates, using a primary antibody and labeled secondary antibody, is described in Steps 24–29. Suggested screening concentrations and antibody dilutions for each test case are provided in **Table 1**. Standard buffers such as TBST or PBST can be used for all experiments.

EQUIPMENT SETUP

Customized microarrayer wash station The standard OmniGrid 100 setup includes a sonicator for aqueous washing of the printing pins. For SMMs, an organic solvent such as acetonitrile is used to wash away the compounds from the pins. The sonication station has been substituted with a stir plate and a recrystallizing dish containing acetonitrile. During each wash step, the printhead is dipped into the stirring acetonitrile dish for 5 s followed by 3 s at the vacuum drying station. For each pin dip, the wash–dry cycle is repeated three times to minimize carryover of samples. Make sure that the stir bar does not create a deep vortex such that the pins do not make contact with solvent. Occasionally monitor the solvent level to ensure that the pins are effectively washed.

Typical GenePix scanner settings Pixel size: 10 μm; photomultiplier tube (PMT) voltages per laser: 635 nm ex. (red) = 500–600; 594 nm ex. (yellow) = 600; 532 nm ex. (green) = 500–550; 488 nm ex. (blue) = 400–500.

PROCEDURE

Preparation of small-molecule stock solutions for printing

1| Dissolve small molecules of interest in DMSO. Typically, printing stock concentrations range from 1 mM to 10 mM. DMF is a suitable alternative solvent for preparing stock solutions. Stock solutions are stored at –20 °C.

2| Transfer 5 μl of each stock solution to individual wells in a 384-well polypropylene microarray plate. For large sample numbers it is desirable to use a liquid transfer robot.

▲ **CRITICAL STEP** Sealed stock plates are stored at –20 °C and undergo up to ten freeze–thaw cycles prior to liquid chromatography–mass spectrometry analysis to monitor the stability of compound stocks. The number of acceptable freeze–thaw cycles often depends on the nature of the small molecules that are printed. A typical set of printing stock plates is retired after twelve freeze–thaw cycles.

Preparation of isocyanate-coated glass microscope slides

3| Place 100 amino-functionalized Corning GAPS II slides into two stainless steel 50-slide racks. Submerge each rack in a large glass trough containing fresh PEG solution: Fmoc-8-amino-3,6-dioxaoctanoic acid (1 mM), PyBOP (2 mM), and DIPEA (0.5 mM) in 1 l of DMF. The solution should completely cover the slides. Incubate the slides in the PEG solution with stirring at room temperature (roughly 24 °C) in a fume hood for at least 4 hours.

■ **PAUSE POINT** The incubation is typically performed overnight.

4| Remove the racks from the PEG solution, and allow them to drip-dry before briefly rinsing in DMF. Drip-dry the racks again, and place them into a clean tank containing 1% (vol/vol) piperidine in 1 l of DMF to remove the Fmoc group from the surface. The deprotection reaction is complete after 10 min at room temperature.

■ **PAUSE POINT** The slides can be left in the deprotection solution overnight.

5| Remove the racks from the piperidine solution, drip-dry, and wash for 1 min in DMF with stirring. To install the isocyanate group on the surface of the slides, place the deprotected slides into troughs containing 1% (vol/vol) 1,6-diisocyanatohexane in DMF. Incubate the fully submerged slides in this solution with stirring for 30 min at room temperature.

6| Immerse the activated slides in DMF with stirring and wash for 3 min. Repeat with fresh DMF. Immerse the slides in THF with stirring and wash for 2 min. This wash sequence effectively removes excess isocyanate reagent from the slides and will provide clean and dry slides.

▲ **CRITICAL STEP** It is important that slides are completely dried prior to printing so that excess solvents and reagents are not exposed to the microarrayer platform. Slides can be dried under a gentle stream of air for a minute or two after the final THF rinse. Otherwise, simply allow the THF to evaporate for a few minutes.

Printing SMMs

7| Remove the compound stock plates from the freezer and allow them to thaw in a desiccator dry storage box.

8| Carefully place the dried and activated slides onto the microarrayer platform. Be sure that the slides are all in a common orientation with respect to the barcode sticker.

9| Load clean printing pins into the printhead being careful to avoid touching the tips of the pins.

10| Design the printing configuration using the OmniGrid 100 software. Printing from DMSO typically provides features with spot diameters of around 150 μm. Using a center-to-center spacing of 300 μm comfortably allows 10,800 features to be printed in 15 × 15 subarrays using 48 pins.

11| Centrifuge all compound stock plates at 400*g* for 1 min at 24 °C using a Genevac HT-24 or standard benchtop centrifuge with microplate adapters.
▲ CRITICAL STEP Plates should be centrifuged to be sure that all of the stock solution resides at the bottom of the well.

12| Insert a clean glass blot pad in one of the three microplate positions. Insert the first two compound stock plates into the remaining microplate holders.
▲ CRITICAL STEP Be sure that all stock plates are placed on the microplate holders in the proper orientation with respect to well A01 to avoid inconsistencies between the actual printing sequence and the theoretical print sequence or GenePix array list (GAL) file.

13| Print compounds in desired array format. Instruct the arrayer to pre-spot 30 features at 400 µm center-to-center spacing on the blot pad for every sample pickup. Clean the blot pad with bibulous paper and methanol after printing every two plates. Printing solutions on a blot pad prior to spotting on the activated slides avoids excess solution from creating large spots on the first few slides of the print run.
? TROUBLESHOOTING

14| After the print run is completed, leave the slides on the microarrayer platform for at least 10 min so that the printed samples will dry.

15| Move printed slides into the stainless steel slide racks. Place the racks in a vacuum desiccator attached to a three-way glass valve through Tygon tubing in a ventilated chemical fume hood. The main outlet should be directed to the desiccator, through tubing, with one of the valves directed to a vacuum line, also through tubing. The other (closed) valve should be directed, through tubing, to a flask with 2 ml anhydrous pyridine. Evacuate the desiccator containing the slides. Keep the slides under vacuum conditions for 5 min to assist the removal of any excess printing solvent. Close off the vacuum line and open the valve to the flask containing pyridine. Next, expose the printed slides in the desiccator to pyridine vapor for at least 2 h. Pyridine catalyzes the covalent attachment of functional groups that are less reactive towards isocyanate. Finally, close off the pyridine line and evacuate the desiccator to dry the slides.
! CAUTION Be sure to handle pyridine in a properly ventilated fume hood to avoid exposure by inhalation.
■ PAUSE POINT The slides are typically exposed to pyridine vapor during an overnight incubation.

16| Remove the racks from the desiccator and immerse the dried slides in a solution of 5% (vol/vol) ethylene glycol and 0.1% (vol/vol) pyridine in DMF with stirring for 30 min to quench the isocyanate surface.
! CAUTION Be sure to wear gloves when removing the racks from the desiccator to avoid exposing skin to the pyridine.

17| After the ethylene glycol quench, rinse the slides in DMF. Wash the slides in DMF for 1 h with stirring followed by two brief washes, 3 min each, in THF. Dry the slides by centrifugation.

18| Dried slides are packaged in five-slide boxes sealed with Parafilm.
■ PAUSE POINT Microarrays can be stored for up to 6 months at –20°C. The arrays can be kept at 4 °C for several days.

Quality control: detecting known protein–small molecule interactions
19| Pre-scan to see known fluors (listed in **Table 1**) and to identify autofluorescent compounds.
? TROUBLESHOOTING

20| Prepare protein or antibody solution to be used to detect a known printed ligand (listed in **Table 1**) in TBST buffer that has been kept chilled at 4 °C. Purified proteins and antibodies are typically screened in the range of 0.1–5.0 µg ml^{-1}.
▲ CRITICAL STEP It is important to use a buffer that is appropriate for the protein of interest. Buffers should contain specific cofactors or reagents that are required for activity or stability. Avoid autofluorescent additives. TBST and PBST are commonly used and provided as examples.

21| Incubate diluted protein with microarray at 4 °C for 1 h. Two incubation methods are described below. The Dish method (A) is used when the protein is not in limited supply or if agitation is desirable (use the same protocol for antibody incubations that might follow). The inexpensive Parafilm method (B) is used to minimize the amount of protein used in the binding assay. This method was used as an alternative to coverslips, which provide inconsistent results and areas of high background surrounding the edge of the coverslip:

(A) Dish method

(i) Place the microarray, printed face up, in the well of a 4-well rectangular dish.
(ii) Gently pipet 3 ml protein solution onto the slide barcode sticker and let the solution spread out to cover the surface of the slide. Alternatively, three slides can be placed printed face up in a square Petri dish.

(iii) Cover the dish with the lid and place on a rocking platform so that the solution is gently agitated over the surface of the slide. Alternatively, gently pipet 6 ml of protein solution into the dish and agitate.

(B) Parafilm method

(i) Cut a strip of Parafilm and place on a smooth and flat surface such as a clean lab bench in a cold room or on a chilled flat surface for transfer into a laboratory refrigerator or cold room.

(ii) Pipet 300 µl of protein solution onto the parafilm.

(iii) Carefully place the microarray, printed face down, onto the drop so that the protein solution spreads out to cover the entire slide.

▲ **CRITICAL STEP** Avoid introducing air bubbles in between the printed surface of the slide and the Parafilm.

22| Carefully remove protein solution from the microarray. Briefly rinse excess protein solution from the slide using chilled TBST buffer (4 °C) using A) for directly labeled fluorescent proteins (e.g. Alexa 647, fluorescein, GFP, etc.) or B) for assays involving detection through a labeled antibody (e.g. anti-His, anti-GST, anti-FLAG, etc.).

(A) Direct detection of fluor-labeled proteins

(i) Wash each slide in 3 ml buffer for 2 min with agitation on a platform shaker or rocker. Repeat twice.

(ii) Wash once with chilled TBS buffer (4 °C) for 1 min and go to Step 23.

(B) Antibody-based detection

(i) Immediately apply the diluted antibody of interest in TBST or another suitable buffer and place the slide at 4 °C for 1 h.

(ii) Carefully remove fluor-labeled antibody solution from the microarray.

(iii) Briefly rinse excess protein solution from the slide using chilled TBST buffer.

(iv) Wash the slide in 3 ml buffer for 2 min with agitation. Repeat twice.

(v) Wash once with chilled TBS buffer for 2 min.

23| Dry slides by centrifugation using a slide centrifuge. The probed microarrays are ready for analysis.

■ **PAUSE POINT** Ideally, slides are scanned immediately after probing with protein. Dried slides can be stored at room temperature and in the dark for up to 2 days prior to scanning without significant deterioration in fluorescent signal.

Protein binding screens using cell lysates

24| Transfect HEK-293T cells with a mammalian overexpression construct encoding an epitope-tagged protein of interest. Cells are seeded in a 6-well plate at 5×10^5 cells per well, anticipating one well will be required per SMM incubation. A reliable, high level of expression has been achieved in this cell line with most commercially available lipid transfection reagents following provided technical protocols. Cells are typically harvested 48–72 h after transfection, the time at which a well that is transfected with an enhanced green fluorescent protein (EGFP) vector achieves a stable, high degree of expression.

▲ **CRITICAL STEP** Protein expression and detection should be validated by immunoblot. Where feasible, immunoprecipitated protein should be assessed for activity in an appropriate biochemical assay.

25| Harvest cells for storage or lysis. Adherent cells are washed twice in chilled PBS in 6-well plates, resuspended in 500 µl of chilled PBS per well and transferred to labeled Eppendorf tubes. Cells are pelleted by brief centrifugation and the supernatant is discarded.

■ **PAUSE POINT** Pelleted cells are typically snap-frozen in liquid nitrogen and stored at –80 °C until use.

26| Prepare cellular lysates for incubation with SMMs. Cell pellets are thawed on wet ice and resuspended promptly and gently in MIPP lysis buffer supplemented with protease inhibitors and fresh DTT (300 µl volume per source well). Incubate on ice for 15 min. Lysates are then clarified by centrifugation at 14,000*g* for 10 min at 4 °C. Immediately following centrifugation, decant supernatant to new, chilled Eppendorf tubes. Perform a protein quantification assay and adjust with lysis buffer to achieve 0.3 µg ml^{-1}.

▲ **CRITICAL STEP** MIPP lysis buffer has been determined to minimize autofluorescence with arrays prepared as above. RIPA lysis buffer interferes substantially with signal-to-noise ratio in controlled experiments.

27| Incubate SMM with lysates using the methods described in Step 21 for 1 h at 4 °C. Wash with gentle rotation in chilled PBST for 1 min, repeating three times.

28| Incubate SMM immediately with primary antibody for 1 h at 4 °C. For epitope-directed antibodies such as anti-FLAG or anti-His, a 1:1000 dilution in PBST supplemented with 0.1% bovine serum albumin (BSA) is suggested. Wash with gentle rotation in chilled PBST (4 °C) for 3 min, repeating three times.

29| Incubate SMM immediately with secondary antibody for 1 h at 4 °C. Dilutions of 1:1,000 are appropriate for most commercial fluor-labeled antibody solutions. Wash with gentle rotation in chilled PBST for 3 min, repeating three times. Briefly rinse with distilled water and dry slides by centrifugation for 1 min. The probed slides are ready for analysis.

Data analysis

30| Scan slides with the GenePix 4200A slide scanner, using the suggested settings.
? TROUBLESHOOTING

31| Align the corresponding GAL file, translating microarray location to microplate location, to each scanned image using the GenePix Pro 6.0 software. Use the printed fluor markers to help align each subarray. Properly resize each GAL file feature to the diameter of the actual printed microarray feature and generate a GenePix results (GPR) file for each microarray.
? TROUBLESHOOTING

32| Analyze results file to evaluate a) whether fluorescent dye markers are present, b) whether known ligands are present, c) whether marker compounds carryover to the next sample resulting in contamination of neighboring features, d) which compounds are autofluorescent at the experimental wavelengths and e) whether there are new small molecules that bind to the protein or antibody applied to the microarray.
? TROUBLESHOOTING

33| Score assay positives from triplicate experimental data, based on deviation from the mock-treatment distribution defined by the features containing solvent only on each SMM. Adjust fluorescence intensity for background signal on a per-spot basis within the GenePix software, and use this metric principally in the analysis.

34| Compare assay positives to triplicate experimental data collected from control experiments as appropriate.
▲ **CRITICAL STEP** As this platform is capable of detecting interactions between small molecules and immunoglobulins, comparison to a buffer-only or control-lysate experiment followed by antibody incubation is essential.

● **TIMING**
Steps 1–2: Time required is dependent on the number of samples to be printed
Step 3: 100 slides, 4.5 h or overnight incubation
Steps 4–6: 1 h
Steps 7–12: 1 h
Step 13: Time required is dependent on the array design
Steps 14–15: Overnight incubation
Steps 16–18: 2 h
Steps 19–23: Protein incubation followed by incubation with antibody, 2.5 h
Step 24: 72 h
Steps 25–29: 6 h
Steps 30–34: 8 h (varies depending on the choice of analytical method)

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Solutions
Step 13	Stock solutions carryover from one sample pick up to the next.	Increase the duration of the pin wash cycle or increase the number of cycles in between each sample pickup. Be sure to clean the blot pad with methanol and bibulous paper after every two plates.
Step 19	Alexa Fluor 647 cadaverine control spots print poorly.	Batch variation for this commercially available fluorescent dye has been observed. Cy5-NHS (GE Healthcare) or VivoTag 680 (Visen Medical) can be used as alternatives.
Step 30	Protein sample gives high background or forms a film across the slide.	A) Avoid adding autofluorescent reagents to the incubation buffer. When in doubt, incubate the buffer of choice with a plain glass slide, dry the slide, and scan to look for the presence or absence of a fluorescent film. B) Test lower concentrations of protein. C) Increase duration of wash in Steps 22 and 23. D) Add 0.1% (vol/vol) bovine serum albumin (BSA) to incubation buffers as a blocking agent.
	Features appear white.	The saturation limit of the scanner has been reached. Lower the photomultiplier tube (PMT) voltage and scan again.
Step 31	GenePix Pro does not properly fit each GAL feature to the actual diameter of the printed feature.	Printed features of irregular size or morphology may require the user to manually fit the GAL file to the feature image.

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Solutions
Step 32	Expected interactions are not witnessed.	If a control interaction is present on the array and not appearing as a clear assay positive, a higher titer of antibody is advisable. Antibodies directed at a protein of interest frequently require a higher concentration of antibody (1:250 to 1:100). Wash with gentle rotation in chilled PBST for one minute, repeating three times.
Steps 19, 32	Printed features are missing.	The stock solution was not properly delivered to the surface of the slide. Make sure that the solution resides in the bottom of the plate and it on the sides of the well. Also be sure to adjust the z-axis so that the pins make contact with solution in each well. Physically inspect the wells to rule out evaporation of the sample. Additionally, check to make sure that all of the pins move freely in the printhead. Also be sure that the pins are properly cleaned. Inspect the pins under a microscope to make sure that the tip is not obstructed or damaged (i.e. bent or corroded).

GAL, GenePix array list file; PBST, Phosphate-buffered saline with Tween-20.

ANTICIPATED RESULTS

Each of the experimental steps outlined in this protocol have been optimized for performance, yield and reproducibility so as to accommodate the fabrication of arrays for screening by a number of interested investigators. However, unanticipated variables introduced by equipment design, operator training and protocol implementation are likely to establish a learning curve during implementation and the need for re-optimization. The ▲ CRITICAL STEP notes and ? TROUBLESHOOTING guide highlight those procedures that require the most careful attention and protocol calibration. Once optimized, the investigator can anticipate the successful immobilization of nearly 11,000 diverse compounds in microarray format on a glass microscope slide. *En route* to this outcome, we recommend 'assay development' screens with fluorescent ligands as controls for the printing process and known high-affinity ligands to validate the platform in a screening context.

The optimized screening protocols for recombinant and transfected protein have proven reliable and robust as described. However, with the testing of new proteins, the influences of proper protein folding and stability in lysis buffer and of the selection of epitope and antibody for detection are substantial. To illustrate the results anticipated from screening SMMs, we present data in **Figure 3** from a screen of a clarified cellular lysate from HEK-293T cells expressing Flag-FKBP12. In this experiment, a primary and secondary antibody detection scheme was used as described above. The array was scanned for fluorescence at 532 nm and 635 nm, false-colored green and red in this merged image, respectively. Assay positives appear in red. These data illustrate the anticipated detection of the small molecule ligand AP1497 (**Fig. 3a**), printed through a primary amine, and the natural product rapamycin (**Fig. 3b**), printed through a secondary alcohol. A histogram depicting the distribution of 635 nm fluorescence intensity corrected for

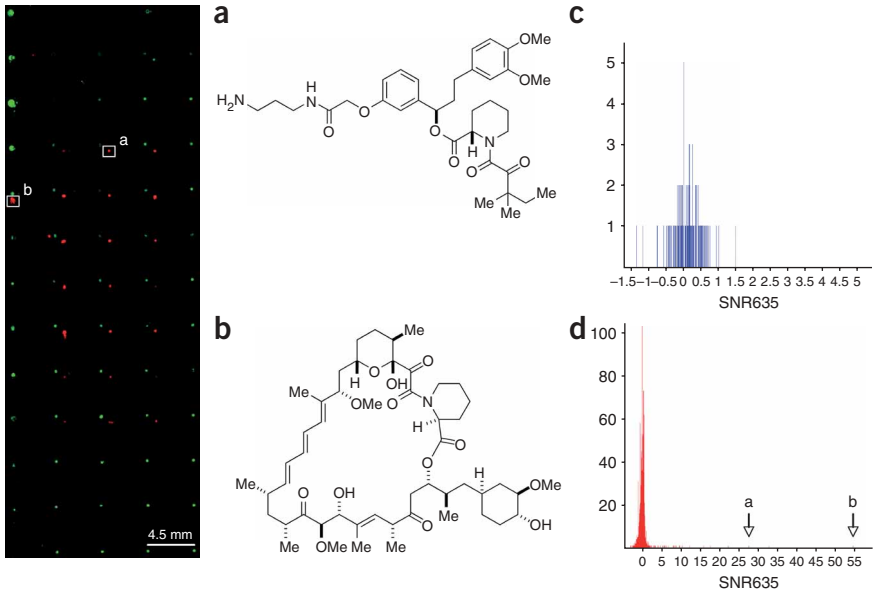

Figure 3 | Small-molecule microarray (SMM) probed with Flag-FKBP12-overexpressing cellular lysates. (**a**) Recognition of an analog of AP1497 printed through a primary amine. (**b**) Recognition of the natural product rapamycin, likely printed through a secondary alcohol. (**c**) Histogram of background-adjusted 635 nm fluorescence intensity data derived from solvent-only features on the SMM. (**d**) Histogram of background-adjusted 635 nm fluorescence intensity data derived from printed small molecule features on the SMM.

local background of wells containing solvent alone is presented in **Figure 3c**, illustrating the low noise of this experiment. A histogram depicting the same measurement from the printed small molecules on the array is presented in **Figure 3d**. This figure illustrates the expected, comparable, low-intensity distribution of signal from inactive compounds and solvent. Additionally, as shown by the data highlighted with arrows, the AP1497 derivative and rapamycin appear as distinct assay positives in this analysis.

In summary, this protocol details an optimized strategy for printing diverse small molecules in microarray format and screening both purified proteins and complex mixtures. Using this platform, we have detected small molecule binders for protein targets with a range of affinities (2 nM to 50 μM), validated by surface plasmon resonance.

Note: Supplementary information is available via the HTML version of this article.

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