Chapter 17

Ligand Discovery Using Small-Molecule Microarrays

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Abstract

Genome-wide association studies and genetic linkage studies have created a growing list of proteins related to disease. Small molecules can serve as useful probes of function for these proteins in a cellular setting or may serve as leads for therapeutic development. High-throughput and general binding assays may provide a path for discovering small molecules that target proteins for which little is known about structure or function or for which conventional functional assays have failed. One such binding assay involves small-molecule microarrays (SMMs) containing compounds that have been arrayed and immobilized onto a solid support. The SMMs can be incubated with a protein target of interest and protein–small molecule interactions may be detected using a variety of fluorescent readouts. Several suitable methods for manufacturing SMMs exist and different immobilization methods may be more or less preferable for any given application. Here, we describe protocols for covalent capture of small molecules using an isocyanate-coated glass surface and detection of binding using purified protein.

Key words: Small-molecule microarrays, Ligand discovery, Isocyanate, Rapamycin, FK506, FKBP12

1. Introduction

Genome-wide association studies have produced an increasing list of proteins that may play a key role in disease. Small molecules may be useful tools in understanding the function of these proteins in a cellular context and in some cases may serve as therapeutic leads. Designing small molecule ligands for proteins in the absence of knowledge about structure or function is a significant challenge. Developing high-throughput functional assays (e.g., enzymatic, DNA-binding, etc.) is also a challenge for protein targets that lack assigned molecular function. High-throughput binding assays may provide a general route toward identifying small molecule probes

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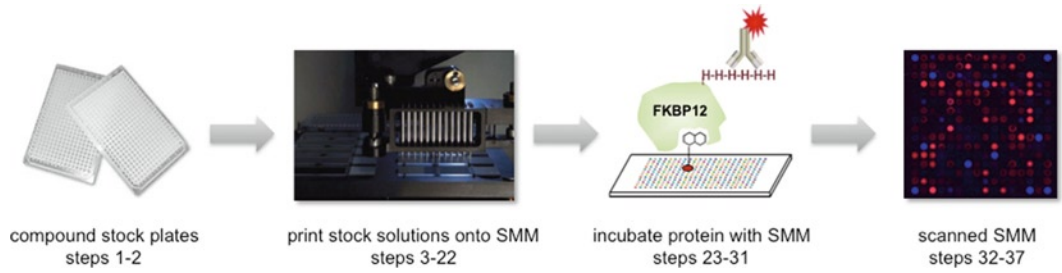

Fig. 1. General scheme for printing and screening small-molecule microarrays (SMMs).

for such targets (1, 2). The small-molecule microarray (SMM) platform has proven to be a robust technology for discovering protein–small molecule interactions (1). The microarray format is particularly attractive for binding assays due to the miniaturized and parallel nature of the format. Typically, more than 10,000 small molecules are evaluated for their ability to bind a protein of interest on a single microarray. Very little compound is required (<200 pL per feature) to make the arrays and tens of thousands of compounds may be screened with as little as 20 μg of protein. SMMs have been used to identify functional probes of many proteins to date including various "druggable" enzymes (3–6), "undruggable" transcription factors (7), and extracellular growth factors (8), as well as nonprotein targets such as pathogenic intron RNA (9).

Several covalent attachment approaches have been developed to immobilize small molecules onto solid substrates such as glass or silicon and have been reviewed elsewhere (1, 10, 11). Most of these capture strategies take advantage of either latent functionalities or specific appendages on the small molecules that react with the modified surface. These approaches can result in either homogenous (5–7, 12) or heterogenous (13–15) display of small molecules. In this chapter, we present an updated step-by-step protocol for manufacture of SMMs using isocyanate-mediated capture and detection of protein–small molecule interactions (Fig. 1). The isocyanate capture approach for small molecules presented here was originally developed by our laboratory in an effort to expand the compatibility of the SMM approach with a wider set of molecules (13, 14). The surface is reactive to compounds containing a variety of nucleophilic functional groups and has been used to immobilize complex natural products, products of diversity-oriented synthesis, drug-like molecules, and known bioactives from commercial collections, and FDA-approved drugs (8, 13). Many compounds contain multiple isocyanate-reactive functionalities, giving rise to the potential for multiple modes of display on the surface. This heterogeneous surface may allow proteins to sample multiple binding modes for any given immobilized small molecule in the array. The surface is also compatible with screens involving pure proteins or proteins residing in complexes or clarified cell lysates. The SMM

fabrication and screening protocols described here serve as a blueprint for building a ligand discovery platform that is general for most types of compounds and proteins.

2. Materials

2.1. Compound Printing and Array Manufacture

1. Amine-functionalized glass slides, GAPSII-Coated Slides (Corning Life Sciences, Inc.).
2. Aushon 2470 Micro-Arrayer (Aushon Biosystems) outfitted with a 48-pin print head and 85 μm diameter solid pins. Other arrayers using split pins ("quill pins") may also be used.
3. Contact Angle Goniometer (VCA Optima XE, AST Products, Inc.) or equivalent.
4. Vacuum Desiccator (VWR International) or equivalent.
5. Glass staining dishes with cover (Wheaton Scientific), or equivalent.
6. Stainless steel slide racks (Wheaton Scientific), or equivalent. Glass slide racks may also be used.
7. Gas filter gun with 0.01 μm PVDF filter (Wafergard GN, Entegris Inc.).
8. Nitrogen gas, ultrahigh purity grade (Airgas, Inc.).
9. Dimethyl sulfoxide, DMSO.
10. Solutions of small molecules (~2.5–10 mM in DMSO) in 384-well V-bottom polypropylene plates (Abgene or Greiner). Typical purity requirements for compounds are ≥ 90%.
11. Dimethylformamide, DMF.
12. FMOC 8-amino-3,6-dioxaoctanoic acid (Chem-Impex International, Inc.). Polyethylene glycol spacers of varying lengths ($n = 2$–10 ethylene glycol units) have been successfully used with this protocol.
13. (Benzotriazole-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate, PyBOP (EMD Chemicals, Inc.).
14. *N*,*N*-Diisopropylethylamine, DIPEA.
15. Piperidine, redistilled.
16. 1,6-Diisocyanatohexane (Sigma-Aldrich).
17. Tetrahydrofuran, THF.
18. Pyridine.
19. (PEO)3-monoamine (Molecular Biosciences, Inc.).
20. Deionized water.
21. HiLyte Fluor 488 amine, TFA salt, fluorescent dye for sentinels (Anaspec).

22. Rapamycin, control ligand to FKBP12 (LC Laboratories).
23. FK506, control ligand to FKBP12 (LC Laboratories).

2.2. Screens with Pure Proteins

1. FKBP12 protein fused to a His tag, for control screen (Abcam).
2. Anti-His antibody, Penta-His Alexa Fluor647 Conjugate (Qiagen).
3. 1× TBST: 50 mM Tris–HCl, 150 mM sodium chloride, 0.1% Tween-20, pH 7.5.
4. 4-Well dish untreated ST lid (Nalge Nunc International).
5. 1-Well dish untreated ST lid (Nalge Nunc International).
6. Rocking platform shakers (VWR).
7. Orbital shaker, works orbital shaker (IKA).
8. Gas filter gun with 0.01 μm PVDF filter (Wafergard GN, Entegris Inc.).
9. Nitrogen gas, ultrahigh purity grade (Airgas Inc.).
10. Distilled water.
11. Microarray scanner, Axon Genepix 4300A Scanner (GENEPIX 4300, Molecular Devices), or suitable equivalent.

3. Methods

Here, we describe an updated protocol for identifying protein–small molecule interactions using SMMs. The protocol was first reported in two manuscripts from our laboratory in 2006 (13, 14). Since that time, we have further optimized selected steps in the SMM manufacture protocol. We also provide updated sources for selected instruments and materials used for both manufacture and screening of SMMs. In this specific example, compounds known to bind to the FKBP12, including the commercially available natural products FK506 and rapamycin, are printed onto isocyanate-coated glass slides. We provide a protocol for detecting interactions of these printed ligands with purified and epitope-tagged FKBP12 as a tutorial for screening (Fig. 1). These interactions are typically used for quality control studies when we manufacture arrays containing screening collections. The ligands described here are typically placed throughout the forty-eight 16 × 16 subarrays on SMMs containing 12,288 printed features. While the protocol described here focuses on the use of a pure protein in the screen, proteins residing in cell lysates may be screened as well. For guidance on screening proteins from cell lysates the reader is referred to the protocol in (14).

3.1. Compound Printing/Array Manufacture

3.1.1. Glass Surface Activation (Fig. 2)

1. Prepare stock solutions of compounds for printing, including dyes for sentinels, FK506, and rapamycin, by dissolving them in DMSO. Typical stocks are prepared in a concentration range of 2.5–10 mM depending upon solubility. Stock solutions are stored at –20°C.
2. Transfer 5 µL of each compound stock solution to individual wells in a V-bottom 384-well polypropylene plate. Plates may be centrifuged before use to eliminate air pockets inside the wells, and insure compound solutions are in the well bottoms.
3. Clean amine-functionalized glass slides prior to printing by using a stream of filtered ultrahigh purity grade nitrogen gas to remove any particulates from the surface. Static contact angle of deionized water on this surface should be 40–45°. Rinse the water from the contact angle test slide with DMF and dry under a stream of ultrapure nitrogen gas.
4. Load amine-functionalized slides carefully into a slide rack. Submerge each rack in a glass staining dish containing fresh PEG linker solution: FMOC-8 amino-3,6-dioxaoctanoic acid (1 mM), PyBOP (2 mM), DIPEA (20 mM) in DMF. The solution should

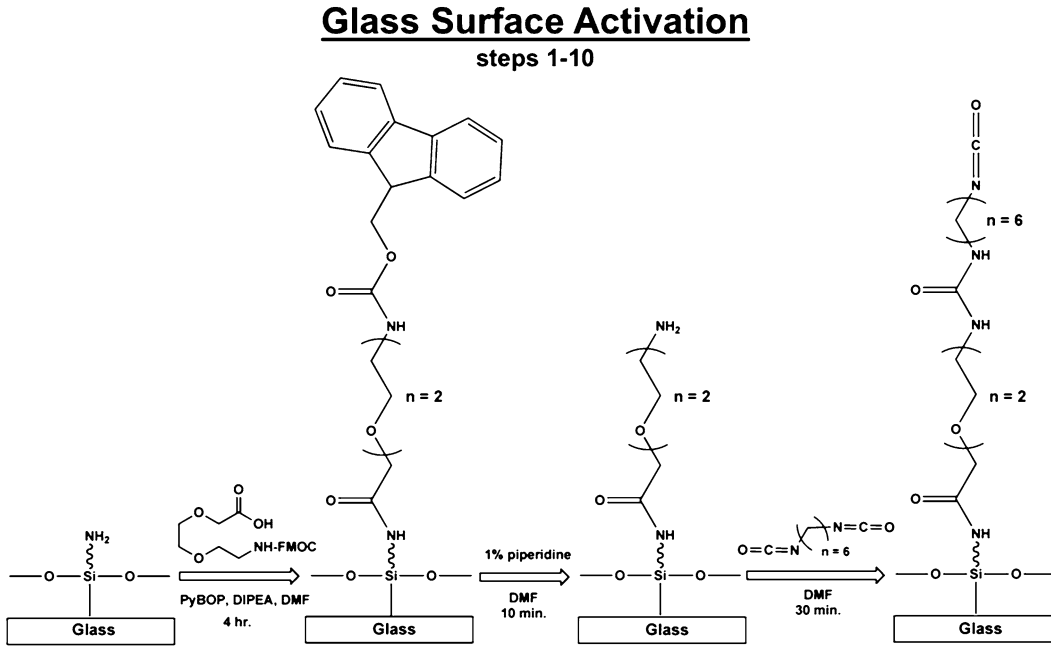

Fig. 2. Scheme for installing a reactive isocyanate group on the surface of a glass slide. Gamma aminopropyl silane (GAPS) slides are coated with an Fmoc-protected polyethylene glycol linker. The protecting group is removed using piperidine, and 1,6-diisocyanatohexane is coupled to the surface to provide the isocyanate glass substrate used in SMM printing. *PyBOP* (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; *DIPEA* *N,N*-diisopropylethylamine; *DMF* *N,N*-dimethylformamide.

completely cover the slides. Incubate the slides in PEG linker solution for at least 4 h (up to 24 h) at room temperature with stirring. Avoid forming a vortex in the stirring solution. Cover the staining dish.

5. Remove one slide from the PEG linker solution, rinse thoroughly with DMF from a squirt bottle and dry under ultrapure nitrogen gas. Static contact angle of deionized water on this surface should be 55–60°. Rinse the water from the contact angle test slide with DMF from a squirt bottle and return to the slide rack.
6. Remove the rack from the PEG linker solution. Briefly rinse slides with a gentle stream of DMF from a squirt bottle, then submerge the rack in a clean staining dish that contains FMOC deprotection solution: 1% (v/v) piperidine in DMF. The solution should completely cover the slides. Incubate the slides in the FMOC deprotection solution for at least 10 min (up to 24 h) at room temperature with stirring. Avoid forming a vortex in the stirring solution. Cover the staining dish.
7. Remove the rack from the FMOC deprotection solution. Briefly rinse slides with a gentle stream of DMF from a squirt bottle, then submerge the rack in a clean staining dish containing DMF, and wash for 1 min with stirring. Avoid forming a vortex in the stirring solution.
8. Remove the rack from the wash solvent, then submerge the rack in a clean staining dish containing diisocyanate solution: 1% (v/v) 1,6-diisocyanatohexane in DMF. The solution should completely cover the slides. Incubate the slides in diisocyanate solution for at least 30 min (up to 2 h) at room temperature with stirring. Avoid forming a vortex in the stirring solution. Cover the staining dish.
9. Remove the rack from the diisocyanate solution. Briefly rinse slides with a gentle stream of DMF from a squirt bottle, then submerge the rack in a clean staining dish containing DMF, and wash for 3 min with stirring. Avoid forming a vortex in the stirring solution. Repeat with fresh DMF for 3 min. Remove the rack from DMF and submerge in THF with stirring and wash for 2 min.
10. Remove the rack from the wash solvent and dry the slides using a stream of filtered ultrahigh purity grade nitrogen gas. Static contact angle of deionized water on this surface should be 40–45°. Rinse the contact angle test slide with THF and dry thoroughly.

3.1.2. Small Molecule Printing (Fig. 3)

1. Isocyanate functionalized slides should either be used for array manufacture immediately or stored under nitrogen or argon gas and desiccated to avoid ambient moisture or other chemical vapors.

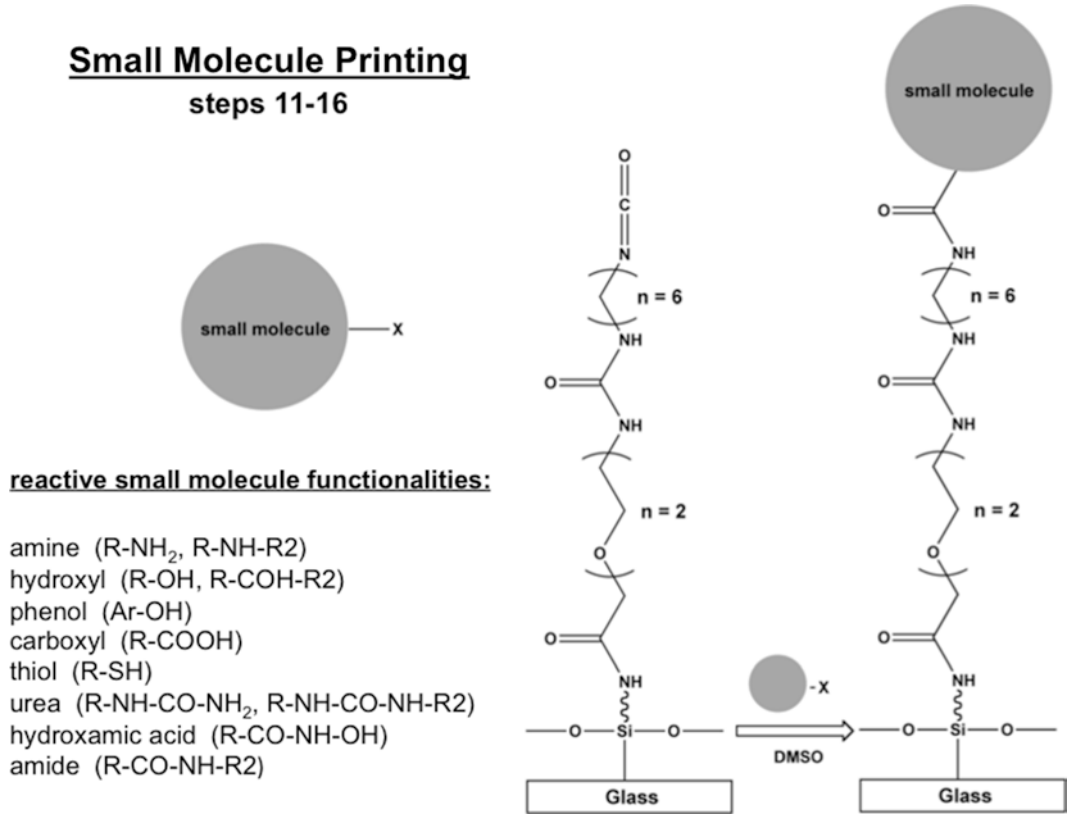

Fig. 3. Activated isocyanate slides are loaded onto the microarrayer platen for small molecule printing. Selected functional groups will react immediately with the glass surface (e.g., amines, thiols, primary alcohols) while less reactive groups require pyridine as catalyst.

2. 384-well V-bottom stock plates containing small molecule solutions should be at room temperature.
3. Arrayer printing pins should be cleaned by sonication in solvents appropriate for removing any trace remains from the last molecule library printed. Typically pins are sonicated in a 50/50 solution of DMSO/water for 20 min followed by 20 min sonication in DMF then 20 min sonication in acetone. Thoroughly dry the pins with a stream of nitrogen prior to printing.
4. Carefully place isocyanate functionalized slides and small molecule stock plates onto the microarrayer platform.
5. Print microarrays using microarrayer and corresponding software. DMSO solutions typically yield 160 μm diameter spots on the isocyanate functionalized glass surface. Typical array feature parameters are set at 270–300 μm feature-to-feature spacing to avoid spots touching or blending together. Pins are washed between each pick up of unique compounds by rinsing

in DMSO five times for 5 s, then touched to bibulous paper to remove the DMSO drop from each pin.

6. When the arrayer run is complete, allow the slides to remain in the arrayer for at least 10 min to allow the DMSO to evaporate from the slide surface.
7. Carefully return the slides to the slide rack. Extra care must be used to not touch or scratch the printed slide surface (see Notes 1–3).

3.1.3. Catalysis and Quench (Fig. 4)

1. Place an open scintillation vial containing 3–5 mL of pyridine inside a vacuum desiccator. Place the slide rack containing arrayed slides in the vacuum desiccator and close. Attach a vacuum

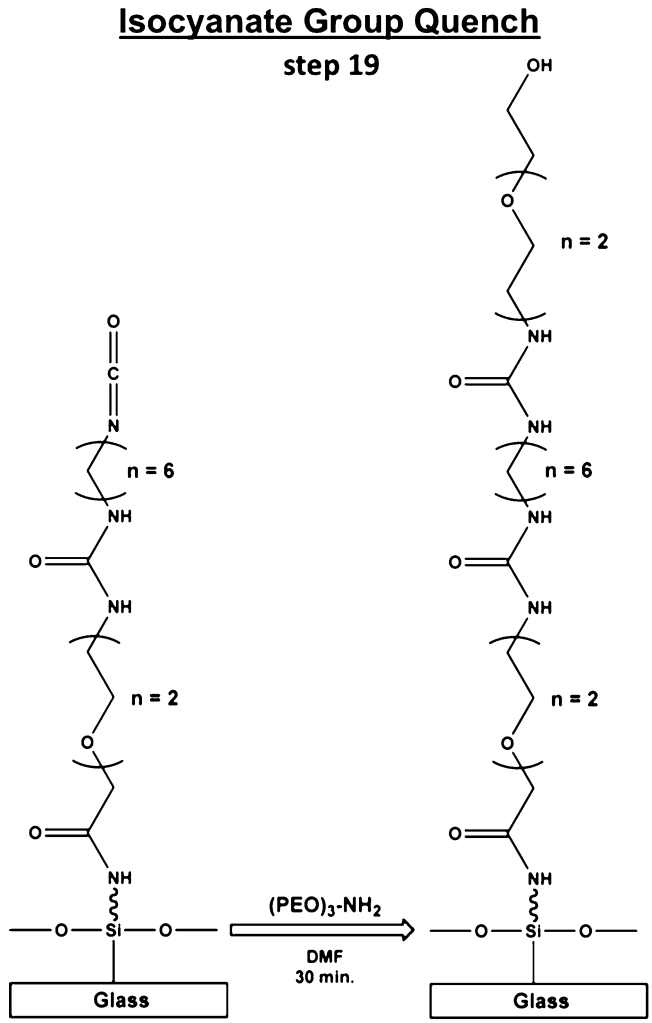

Fig. 4. Unreacted isocyanate groups on the slide surface are quenched with (PEO)3-monoamine to avoid covalent attachment of proteins during a screen. Failure to create an inert background surface may result in very high-background fluorescence on the slide.

hose and pump and evacuate the desiccator. This will create a pyridine vapor inside the desiccator chamber. Allow the slides to remain in the vacuum chamber exposed to the pyridine vapor for 24 h. Pyridine catalyzes the covalent attachment of functional groups that have low reactivity to isocyanate. The vapor pressure of pyridine at room temperature is 18 mmHg.

2. Remove the slide rack from the desiccator and submerge in a clean staining dish containing EG3 quench solution: (PEO)3-monoamine (10 mM) in DMF. The solution should completely cover the slides. Incubate the slides in EG3 quench solution for at least 30 min at room temperature with stirring. Avoid forming a vortex in the stirring solution. Cover the staining dish.
3. Remove the rack from the EG3 quench solution. Briefly rinse slides with a gentle stream of DMF from a squirt bottle, then submerge the rack in a clean staining dish containing DMF, and wash for 1 h with stirring. Avoid forming a vortex in the stirring solution. Remove the rack from DMF and submerge in THF with stirring and wash for 3 min. Repeat with fresh THF for 3 min.
4. Remove the rack from the wash solvent and dry the slides using a stream of filtered ultrahigh purity grade nitrogen gas. Static contact angle of deionized water on this surface should be 40–45°. Rinse the contact angle test slide with THF and dry thoroughly (see Notes 1 and 2).
5. Dry arrayed slides may be stored under inert gas in sealed containers at −20°C for up to 6 months if they will not be used in binding assays immediately.

3.2. Screens with Pure Proteins: Detecting Known Interactions with Fkbp12

1. Prepare 6 mL of FKBP12 protein solution (1 μg/mL) for each microarray slide in TBST. Most proteins are typically screened at concentrations in the 0.1–2 μg/mL range. TBST is a good general binding buffer for many proteins but we typically choose an appropriate buffer for each protein on a case-by-case basis. For example, buffers should contain specific cofactors to maintain appropriate protein conformation or activity when necessary. Care should be taken to avoid autofluorescent additives to any buffers used in the protocol. Insert the microarray slide, printed face up, in one well of the four-well dish.
2. Add 6 mL of the protein solution from the bottom right corner of the well while manually rocking the dish back and forth. Take caution to avoid air bubbles.
3. Place dish with slide on a rocking platform and allow protein binding to occur for 30 min at room temperature. Note that thermally sensitive proteins may be incubated with the microarrays at 4°C for 60 min. Be sure to use chilled buffers for all applications involving thermally sensitive proteins.

4. After the incubation, remove slide from the four-well dish and place it in a single well dish containing TBST buffer. Place on orbital shaker for 1 min and repeat the rinse two times.
5. Prepare a 6-mL solution of a 1:1,000 dilution of anti-His antibody in TBST, and dispense solution in one of the wells of a four-well dish.
6. Place washed slide in antibody solution, printed face up, and incubate while on a rocking platform. Allow antibody binding to occur for 30 min at room temperature. As mentioned previously in step 4, alternative conditions may be used for thermally sensitive proteins.
7. After incubation, remove slide from the four-well dish and single well dish containing TBST buffer. Place on an orbital shaker for 2 min. Repeat the wash step twice using TBST. Perform one final wash in TBS buffer.
8. Perform a final brief 10-s rinse in distilled water with gentle manual agitation by gently rocking the dish back and forth.
9. Dry slides by centrifugation or under a gentle stream of nitrogen gas. Ideally slides are scanned for fluorescence immediately after drying. When using most fluor-conjugated proteins or antibodies, dried slides may be stored at room temperature and in the dark for up to 2 days prior to scanning without significant deterioration of fluorescent signal.
10. Place dried microarray in scanner. Scan the array using the 635 and 488 nm lasers. Adjust the PMT voltages appropriately to avoid features with saturated signal. See Fig. 5 for a scanned array image (see Notes 4 and 5).
11. Align the corresponding GAL file for the microarray print run onto the scanned image using the GenePix Software. Printed fluorescent sentinels (488 nm) are used to generally align the grid and the diameters of the grid features are fit to the fluorescent features using the automated feature in the software. Manually inspect the fitted GAL grid to be sure that the software has sized the features appropriately (see Note 6).
12. Using the Genepix software, generate a GPR file that contains information about fluorescent intensity, signal-noise-ratios, spot diameter, and other types of information for the scanned microarray.
13. Examine GPR data to determine whether known ligands to FKBP12 have been detected. Check to see whether compounds have carried over to the next sample pickup resulting in contamination of the next printed feature.

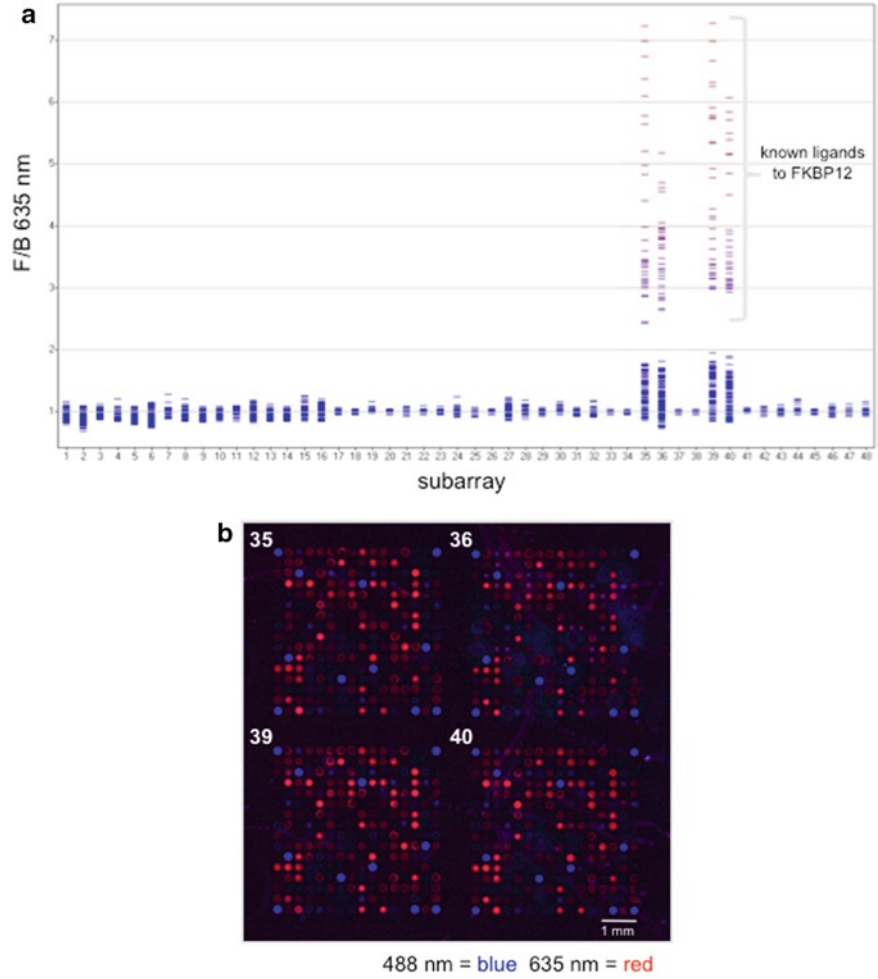

Fig. 5. Small-molecule microarray (SMM) probed with pure FKBP12-His6 followed by an Alexa647-labeled anti-His antibody. (**a**) Plot of foreground signal over local background signals (F/B) for compounds printed in each of the 48 subarrays. Each feature has a local background set to three times larger than the feature diameter. The typical SMM contains 48 subarrays, each with 256 printed features in a 16×16 array configuration. Subarrays 35, 36, 39, and 40 contain positive control ligands to FKBP12. All of the ligands show higher F/B values than the other printed features. (**b**) *Inset* image of subarrays 35, 36, 39, and 40. Signal at 488 nm corresponding to the control fluorescein dye features used for aligning the GAL file grid is false-colored *blue*. Signal at 635 nm corresponding to features where the Alexa647-labeled antibody is bound to the SMM is false-colored *red*.

14. Using data contained within the GPR file, compute foreground signal over local background (F/B) values where each feature has a local background that is set to three times the feature diameter (Fig. 5).
15. Compare data from replicate screens. Most screens are run on three replicate slides.

4. Notes

1. In case of a problem with array features blending together:
   (a) Source plate contaminated with solvent. Solvent contamination (e.g., methanol, ethanol, etc.) in the DMSO solution source plates can lead to changes in spot morphology and typically results in spreading of the spot diameter.
   (b) Arrayer pins are not clean. Contamination of pins with dust or precipitated compound may result in pin tips with artificially greater surface area, resulting in pick up and deposition of larger volumes of compound stock solution. Thoroughly clean pins by sonication as described, or remove contaminated pins and gently clean with a cotton swab and solvent. *It is critical not to use cotton swabs that contain adhesive or plastic.*
   (c) Isocyanate surface activation reaction errors. If static water contact angles are not within the range described in the protocol, it is likely the surface chemistry activation reactions have failed. Use fresh reagents, and insure no water or other contamination occurs to the reagents.
   (d) Arrayed slide is not allowed to thoroughly dry. Printed arrays must be allowed to thoroughly dry before removal from the arrayer. The volume of each printed spot should be small enough that 10 min is enough time to allow the plate to dry after printing. No artificial humidification should be supplied to the printing atmosphere.
   (e) Printed features are too close together. Aim to leave at least 100 μm spacing between spots, larger volume spots may require more distance. Also program the arrayer to place successive spots at least two rows and columns away from the most recent printed spot if possible.
   (f) Arrayed slides kept in pyridine vapor too long or vacuum pressure too low. Some compound spots may increase in diameter if kept under vacuum too long or under vacuum that nears the vapor pressure of the compound. The pyridine vapor catalysis step should be done under vacuum not below 17 mmHg.
2. In case of a problem with array spots missing:
   (a) Stock solution not delivered to the glass slide. Check that the source plate contains solution at the bottom of the wells in sufficient volume and that the source plate solutions have not evaporated. Ensure that the arrayer print head is adjusted in the *z*-axis so that pins are contacting the source plate solutions as well as the glass surface.

Inspect and clean any pins that show contamination; "quill" type pins may become obstructed with compounds that have dried on the pins. Also inspect pins to insure they are not damaged or bent.

(b) Compound is not reactive. Selected compounds may not contain a functional group that covalently reacts with an isocyanate. Nonreactive compounds typically rinse away during the EG3 quench reaction, and those spots will react with the (PEO)3-monoamine quenching reagent. Selected compounds that lack an isocyanate-reactive group will nonspecifically stick to the surface. In our experience, some flat and hydrophobic compounds that resemble dyes will stick nonspecifically.

(c) Pyridine catalysis step fails. Ensure the vacuum desiccator is completely sealed and vacuum is maintained to create a pyridine vapor atmosphere.

3. In case of a problem with compound solution carryover from one sample pick up to the next:

(a) Pins are not clean. Increase the duration and number of pin wash cycles between each unique sample pick up.

(b) Pin blotter is contaminated. If a blotter is used to remove solvent from the pins after cleaning, change or clean the blotter between print runs.

(c) Wash solution is contaminated. Replace wash solution with fresh solvent.

4. In case of high-background fluorescence across array:

(a) Avoid autofluorescent additives in buffers. The reagents may form a fluorescent film across the slide that obscures signal from binding interactions. When in doubt, a plain glass slide may be incubated in the buffer of interest, dried and scanned for fluorescence.

(b) Protein concentration is too high. High concentrations of protein (>5 μg/mL) may lead to film formation on the surface. Reduce the amount of protein or evaluate a series of concentrations to optimize F/B values.

(c) Protein is denatured. In our experience, denatured protein samples often display higher background binding to the array surface relative for native protein samples.

(d) Increase duration of washes after final antibody incubation. Extra washes may reduce binding to array background as well as printed small molecules.

(e) Preblock arrays prior to incubation with protein. Although not required for most proteins, blocking agents such as bovine serum albumin (BSA) or SynBlock (AbD Serotec)

may be used to reduce background binding. Slides may be preblocked in SynBlock solution 0.1% (v/v) BSA in the appropriate incubation buffer for 60 min prior to incubating with protein. BSA may also be used in subsequent incubation steps.

5. In case that scanned features appear white: The saturation limit of the scanner has been reached. Lower the PMT voltage on the scanner and rescan.
6. Genepix Pro does not properly autofit each printed feature using the GAL file. Selected printed features of irregular shape or size may require manual fitting of the GAL file to the printed feature image.

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