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Research Paper

A one-bead, one-stock solution approach to chemical genetics: part 2

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Abstract

Background: Chemical genetics provides a systematic means to study biology using small molecules to effect spatial and temporal control over protein function. As complementary approaches, phenotypic and proteomic screens of structurally diverse and complex small molecules may yield not only interesting individual probes of biological function, but also global information about small molecule collections and the interactions of their members with biological systems.

Results: We report a general high-throughput method for converting high-capacity beads into arrayed stock solutions amenable to both phenotypic and proteomic assays. Polystyrene beads from diversity-oriented syntheses were arrayed individually into wells. Bound compounds were cleaved, eluted, and resuspended to generate 'mother plates' of stock solutions. The second phase of development of our technology platform includes optimized cleavage and elution conditions, a novel bead arraying method, and robotic distribution of stock solutions of small molecules into 'daughter plates' for direct use in chemical genetic assays. This library formatting strategy enables what we refer to as annotation screening, in which every member of a library is annotated with biological assay data. This phase was validated by arraying and screening 708 members of an encoded 4320-member library of structurally diverse and complex dihydropyrancarboxamides.

Conclusions: Our 'one-bead, multiple-stock solution' library formatting strategy is a central element of a technology platform aimed at advancing chemical genetics. Annotation screening provides a means for biology to inform chemistry, complementary to the way that chemistry can inform biology in conventional ('investigator-initiated') small molecule screens.

Keywords: Cell-based assay; Chemical genetics; Diversity-oriented synthesis; Fluoridolysis; Small molecule microarray

1. Introduction

The process of discovering and using small molecules that bind specifically to proteins and alter their functions can be subdivided into forward and reverse chemical genetic approaches. By analogy to classical genetics, forward chemical genetics refers to the observation of a small molecule-induced phenotype followed by the identification of the protein to which it binds. Reverse chemical genetics, analogous to a 'knockout' experiment, refers to the identification of a small molecule that binds to a particular protein, followed by the targeted modulation of function by the small molecule and the subsequent search for phenotype. In recent years, both approaches have been vali-

Abbreviations: BrdU, 5-bromodeoxyuridine; CH_2Cl_2, dichloromethane; CH_3CN, acetonitrile; DMF, dimethylformamide; DMSO, dimethylsulfoxide; HF, hydrogen fluoride; HF/py, hydrogen fluoride/pyridine; HF/Et_3N, hydrogen fluoride/triethylamine; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LC/MS, tandem liquid chromatography/mass spectroscopy; MS, mass spectroscopy; NMR, nuclear magnetic resonance; PS, polystyrene; TBAF, tetrabutylammonium fluoride; THF, tetrahydrofuran; TMSOMe, methoxytrimethylsilane; UV, ultraviolet

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dated by important discoveries. A forward chemical genetic approach was used to discover monastrol, a specific inhibitor of the mitotic kinesin Eg5 [1]. Using small molecule microarrays [2,3], uretupamine was shown to bind the yeast protein Ure2p, and subsequently was used to probe a glucose-sensing signaling pathway in yeast (F. Kuruvilla, S.M. Sternson, P.J. Hergenrother, A. Shamji, and S.L.S., submitted). While both discoveries highlight the ability of chemical genetics to study biology, neither was designed to address global features of the interaction of small molecules with biological systems. Typically, assays are performed to illuminate biology, and continue only until the discovery of a useful probe. In contrast, annotation screening aims to associate each product of a diversity-oriented synthesis pathway with a dataset delimiting its biological performance. This goal is analogous to obtaining an NMR spectrum for each compound, and requires that annotation screens include every compound produced by a given synthetic effort. Chemical genetics aims to explore biology systematically, and in particular to generate data architectures that allow emergent properties of both chemistry and biology to be recognized and exploited.

Split-pool [4,5] diversity-oriented synthesis [6] and solid-phase purification [7] provide for the efficient synthesis of structurally complex and diverse compounds for chemical genetic research. Not yet available, however, is a general method to deliver these compounds efficiently into multiple phenotypic [8] and protein-binding [2,3] assays. Critical to this development are optimization of compound cleavage and elution [9], maintenance of the positional integrity of library compounds throughout the formatting process [10], and a means of informing future synthetic efforts with biological assay results. Our early experience [11] using 90 μm TentaGel beads [12], to which compounds were attached via a photolabile linker [13], uncovered a number of limitations at the compound screening stage [10]. In particular, we concluded that efficient screening requires a library realization platform that produces a minimum of 50 nmol/bead. To this end, we adapted polystyrene (PS) macrobeads for diversity-oriented syntheses that yield at least 100 nmol of compound per bead [14]. Small molecules are synthesized on the large beads, which serve as individual reaction vessels during split-pool library synthesis, ultimately delivering ~5 mM stock solutions arrayed into high-density microtiter plates [9]. In the preceding paper in this issue, we reported the first phase of development of a two-part 'one-bead, one-stock solution' technology platform, including the scaled synthesis of a high-capacity solid-phase bead/linker system and the development of a reliable library encoding/decoding strategy [15].

Here we report the second phase of development of this platform, including (1) bead arraying, (2) automated compound cleavage, elution, and resuspension as spatially arrayed stock solutions, and (3) assay format, design, and annotation. Systematic library formatting and annotation screening are illustrated in Fig. 1. We used a small number of model compounds to optimize the cleavage and elution reactions and assess the fidelity of our arraying protocol, evaluating quantitatively several experimental parameters independently of particular chemistry or reaction pathway development. We hope to provide a 'best practices' approach for bridging diversity-oriented synthesis with high-throughput biological assays, with the goal of making chemical genetics a portable and accessible vehicle to the chemist interested in exploring biology. The second phase of development of our technology platform was validated by library formatting and annotation screening of 708 beads from an encoded 4320-member library of structurally complex dihydropyrancarboxamides (**10**). In particular, it is desirable to perform multiple phenotypic and protein-binding assays on the same ordered collection of small molecules resulting from diversity-oriented synthesis. We expect feedback from comprehensive screening efforts to enable new insights into the relationship between the structure of a small molecule and its tendency to modulate biological systems.

2. Results and discussion

2.1. Development of an optimization assay

The principal objective of our technology platform is to deliver quantities of compounds from single beads that are compatible with numerous chemical genetics assays. In general, we perform fluoridolysis to cleave silyl ether-linked small molecules from our trialkylsilyl linker system (**1**) [14,15], and subsequently elute released compounds trapped in the interior of 500–600 μm PS beads. To optimize the conditions for compound release, we elected first to optimize the cleavage reaction and compound elution steps manually, allowing us to avoid extensive reprogramming of our robotics workstations to handle the large number and range of experimental variables. To ensure standardization of these experiments, we prepared three large batches of test resin that required minimal handling prior to compound loading.

Using 'off-the-shelf' beads prepared as described previously [14] and in the preceding paper in this issue [15], we activated diisopropylalkylsilyl-functionalized resin **1** containing ~200 nmol of Si/bead by treatment with excess triflic acid (TfOH) [16] to form **2**, then trapped primary, secondary, and phenolic alcohols **6**–**8** in the presence of excess 2,6-lutidine to generate silyl ethers **3**–**5** (Fig. 2). Naphthyl derivatives **6**–**8** were chosen on the basis of their ease of quantitation by ultraviolet (UV) spectrophotometry and high-performance liquid chromatography (HPLC). To facilitate quantitative measurements of yield from individual PS beads, we first used pure samples of **6**–**8** to establish standard curves of UV peak area (224 nm) versus concentration for **6**–**8** using our HPLC system. In addi-

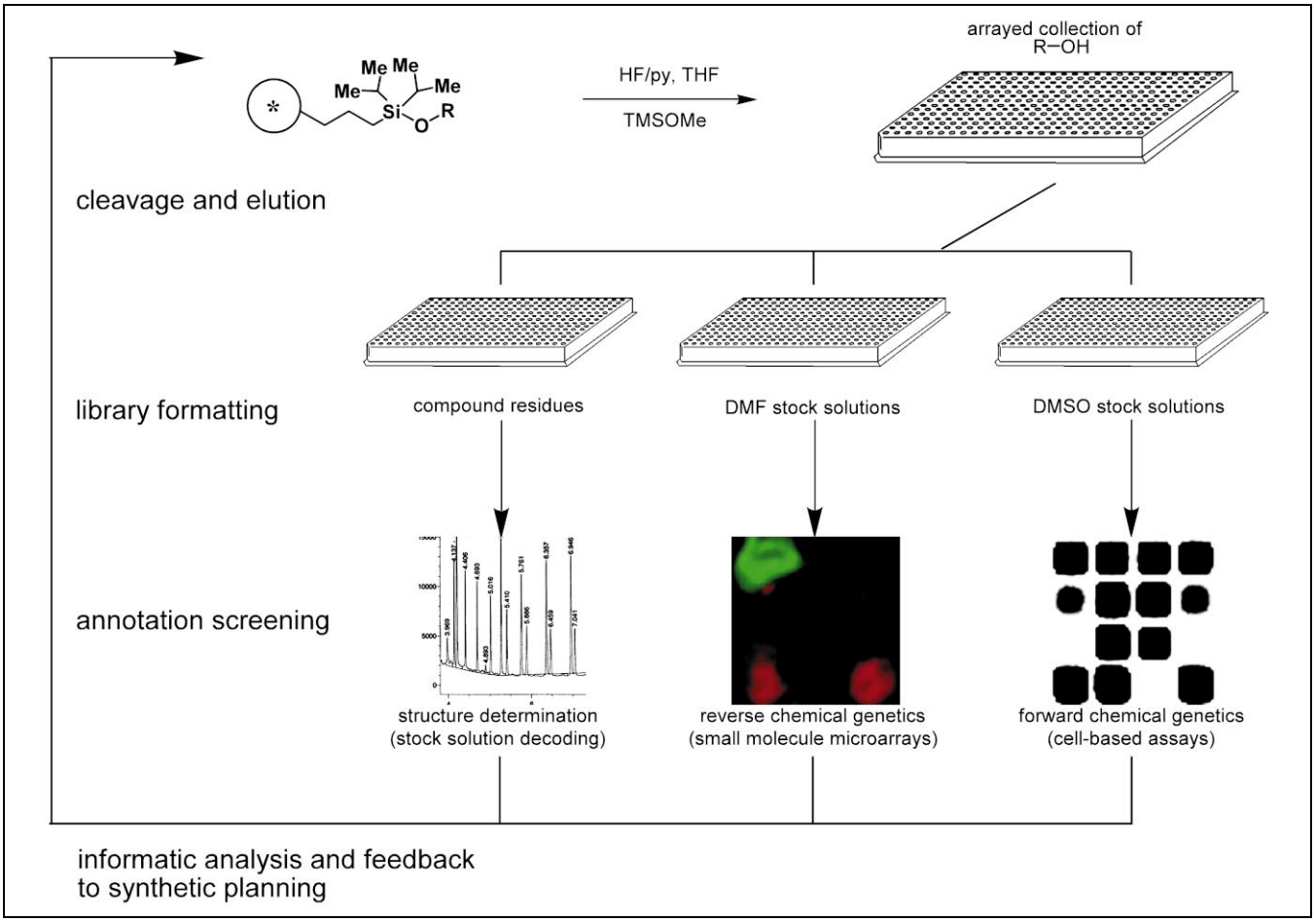

Fig. 1. Overview of library formatting and annotation screening.

tion, we performed mock cleavage reactions on **3**–**5** to determine the lower limit of detection. For each model alcohol, we can quantitatively detect 1–30 nmol of injected compound using our instrumentation.

2.2. Optimization of compound cleavage conditions

The use of silyl ethers to attach alcohols to solid support [17,18] is inspired by the widespread use of silylation as a protection strategy [19]. Fluoride is a reagent of choice for the cleavage of silyl ethers, resulting in fluoridolysis of the Si–O bond and generation of the corresponding silylfluoride. Previously, we have cleaved compounds from **1** primarily with hydrogen fluoride/pyridine (HF/py) solution [14,15,20], a standard reagent for fluoridolysis [19] that is available in a commercial preparation. Excess HF in cleavage reactions is quenched with methoxytrimethylsilane (TMSOMe) [21], resulting in the production of volatile by-products (methanol and trimethylsilylfluoride) that are easily removed from the mixture under vacuum. Before optimizing the cleavage reaction itself, we determined how quickly the addition of TMSOMe results in the quenching of excess HF. To our knowledge this has not been tested explicitly, and is important to establish the timing of an automated cleavage and elution process.

We performed ^{19}F NMR experiments on simulated cleavage reaction mixtures. We added 20 μl of a tetrahydrofuran (THF) solution containing 5% (v/v) HF/py and 5% (v/v) additional pyridine to individual wells of a 384-well plate, each containing a single bead from resin batch **3**. Each reaction mixture was treated with 20 μl of either TMSOMe or *n*-Bu_2O, and immediately transferred to a lined glass NMR tube for determination of the ^{19}F NMR spectrum. The spectrum of samples treated with TMSOMe consists of a singlet at $\delta = 52.4$ ppm, which corresponds exactly to the singlet in the ^{19}F NMR spectrum of an authentic solution of trimethylsilylfluoride in THF. *n*-Bu_2O was chosen as a control because its density (0.764 g/ml) is similar to that of TMSOMe (0.756 g/ml). The spectrum of samples treated with *n*-Bu_2O consists of a broad singlet at $\delta = 125.3$ ppm, and is identical to the spectrum of an authentic solution of HF/py alone. We conclude that HF/py is quenched within 5 min of the addition of TMSOMe as described. In addition to the timing of cleavage and elution, this experiment establishes an important safety margin for the handling, especially

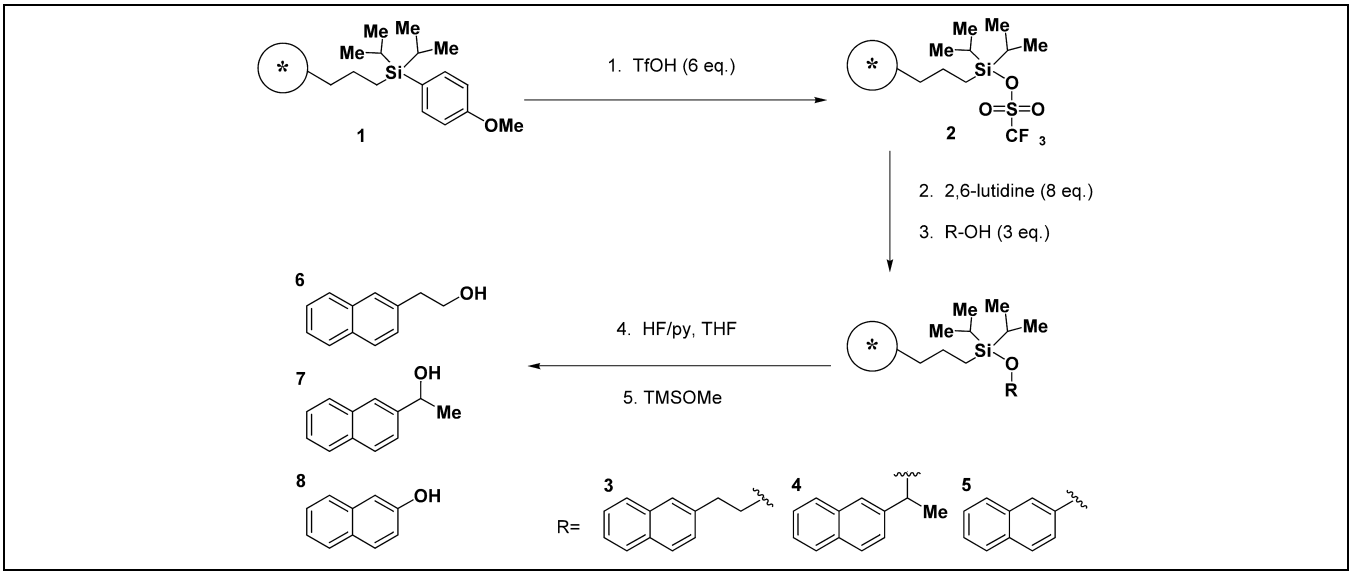

Fig. 2. Loading scheme for model compounds. Model alcohols **6**–**8** were loaded onto PS beads functionalized with trialkylsilyl linker **1**. Model resins **3**–**5** were used for all experiments to optimize the cleavage and elution process. Fluoridolysis reactions yield the starting alcohols **6**–**8**.

robotically, of microtiter plates containing quenched HF/py solutions.

Although multiple reports exist [14,15,18,20–22] of HF/py-mediated cleavage of resin-bound silyl ethers, to our knowledge no solid-phase study has been performed using a standardized system that addresses all experimental variables for this reaction. We systematically tested the composition of the cleavage cocktail, including the identity and concentration of the cleavage reagent, as well as the kinetics of the cleavage reaction. Although these optimization experiments were performed manually, we sought to emulate closely the experimental conditions we envisioned for automated cleavage of compounds. Cleavage experiments were carried out manually by arraying individual beads from resin batches **3**–**5** into single wells of 384-well microtiter plates. At this stage, all exposures of beads **3**–**5** to cleavage reagents took place in 20 μl of THF solution containing a cleavage reagent and a buffering base, as required. After addition of the cleavage cocktail, plates were covered by an empty microtiter plate to prevent evaporation and allowed to incubate at room temperature for the duration of the experiment. Individual cleavage reactions were quenched by addition of 20 μl TMSOMe for an additional 10 min. After evaporating quenched reaction mixtures, we eluted products **6**–**8** by washing each bead twice with 20 μl acetonitrile (CH_3CN) and pooling the two eluates. Calculation of per-bead yield was performed by HPLC analysis as described above.

To select a cleavage reagent, individual beads from resin batches **3**–**5** were exposed for 30 min to a 5% (v/v) solution in THF of either HF/py, HF/triethylamine (HF/Et_3N), aqueous HF, or tetrabutylammonium fluoride (TBAF). In addition, three buffered cocktails were tested: HF/py buffered with 20% additional pyridine, HF/Et_3N buffered with 20% additional Et_3N, and aqueous HF buffered with 20% 2,6-lutidene. As the data suggest, HF/py is four times as effective as any other reagent in releasing **6**, all other factors being equal (Fig. 3a). Similar results were obtained with secondary alcohol **7** (Fig. 3b), though with significantly reduced yields, which we believe are partially due to a lower loading level for **4** (data not shown), and partially due to the short time of exposure, a difference previously observed between primary and secondary silyl ethers [19]. Notably, TBAF performed as well or better than any of the acidic fluoride reagents in the release of **8** (Fig. 3c). Furthermore, for each HF reagent tested, cleavage of **5** to yield **8** was markedly enhanced in the presence of additional buffering base. Taken together, these data suggest that HF/py, buffered with additional pyridine, is the most general cleavage reagent for this system. We suggest, however, that TBAF, or HF solutions buffered with bases other than pyridine, might prove slightly more useful for cleaving phenolic silyl ethers.

Having chosen HF/py as our 'best practices' cleavage reagent, we tested whether the amount of additional buffering pyridine was important to the yield of the cleavage reaction. Once again, this parameter proved sensitive to the type of silyl ether linkage. For resin **3**, loaded with primary alcohol **6**, no significant difference was detected up to 20% additional pyridine (20.0 ± 3.2 nmol/bead). However, both resin **4** and resin **5** were sensitive to the amount of buffering base. Resin **4** showed a marked decline in the yield of **7** as buffering pyridine was increased from 5% (3.6 ± 0.3 nmol/bead) to 20% (2.0 ± 0.3 nmol/bead). In contrast, but consistent with our earlier observation, resin **5** showed a monotonic increase in the yield of **8** as buffering pyridine was increased from 5% (24.3 ± 2.6 nmol/bead) to 20% (30.3 ± 0.6 nmol/bead). Furthermore, in the absence of buffering pyridine, the yield of **8** was only half-maximal (14.1 ± 6.1 nmol/bead). Because our

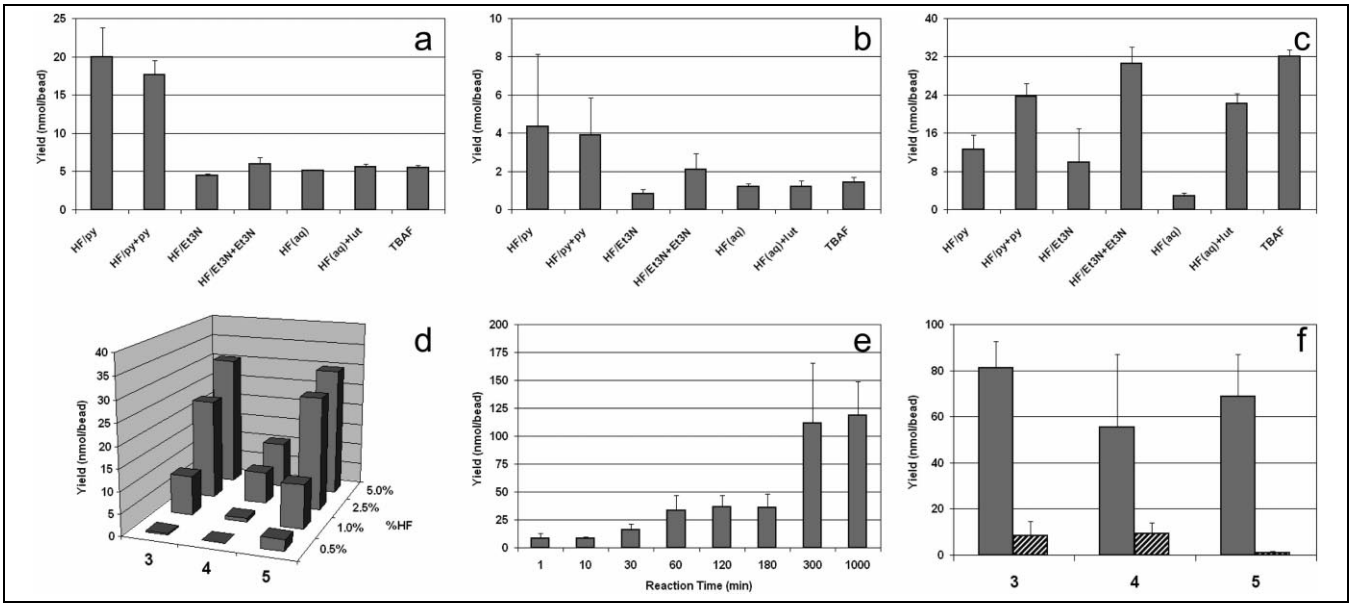

Fig. 3. 'Best practices' cleavage reaction. a–c: Selection of cleavage reagent. Resins **3**–**5** were treated for 30 min with a 5% solution of the indicated cleavage reagent, and (where indicated) additional buffering base, to yield **6**–**8**. Yields represent nmol/bead for (a) primary alcohol **6**, (b) secondary alcohol **7**, and (c) phenolic alcohol **8**. Data are plotted as the mean ± S.D. for eight independent reactions. d: Dependence of yield on %HF. Resins **3**–**5** were treated for 120 min with HF/py+py to generate **6**–**8**. Yields represent mean nmol/bead for six independent reactions. e: Kinetics of fluoridolysis. Resin **3** was treated for the indicated time with HF/py+py to generate **6**. Data are plotted as the mean ± S.D. for 12 independent reactions. f: Double exposure to cleavage cocktail. Resins **3**–**5** were twice (successively) treated for 300 min each with HF/py+py to generate **6**–**8**. Yields represent independent quantitation of the first (solid bars) and second (hatched bars) exposures to cleavage reagent. Data are plotted as the mean ± S.D. for six independent reactions.

HPLC eluent contains trifluoroacetic acid, we believe this effect is indeed a consequence of poorer cleavage, rather than an increase in absorbance of **8** due to deprotonation at the detection step. For generality, we used 5% buffering pyridine in all further experiments. In no case were yields using this cocktail lower than 80% of the maximal yield in any parallel buffering experiment. It should be noted, however, that additional amounts of buffering pyridine might significantly improve the release of phenolic silyl ethers.

We tested whether decreasing the amount of HF in the cleavage reaction would compromise compound yields. Since HF, even in solution, is both corrosive and highly toxic, it would be prudent to use the minimum acceptable concentration, especially when implementing our strategy robotically. To test this parameter, resins **3**–**5** were exposed for 120 min to varying concentrations of HF/py, each buffered with 5% additional pyridine, in THF. Not surprisingly, all lower concentrations of HF resulted in lower yields for all three model compounds (Fig. 3d). Additional experiments with higher concentrations of HF/py suggested that little additional yield was accessible by raising the HF concentration, though 10% HF/py did increase the rate of cleavage of **4** to **7** modestly (data not shown). Thus, a THF solution containing 5% commercial HF/py, plus 5% additional pyridine (HF/py+py), proved most general for liberating **6**–**8** from trialkylsilyl linker **1**.

Despite the optimization of our cleavage cocktail, the absolute yields in the experiments described above fall short of our goal of 50 nmol/bead. To improve these yields, we subjected individual beads from resin batch **3** to our 'best practices' cleavage cocktail for increasing reaction times. In contrast to our past experience [9,14], this experiment demonstrated that the release of alcohol **6** proceeds well past the 120 min mark in the cleavage reaction (Fig. 3e). Indeed, we judged the reaction to be complete only after 300 min, as evidenced by the fact that an overnight reaction did not further increase the yield. This optimization step proved very important to achieving a high yield of **6** (>100 nmol) from large PS beads. Finally, a recent study suggests that multiple exposures to HF/py, presumably with an intervening elution step [22], might also increase the yield of silyl ethers cleaved from solid support. To test this possibility, we first exposed resins **3**–**5** to HF/py+py cocktail for 300 min, after which compounds **6**–**8** were eluted normally (2×20 μl CH_3CN) from the beads. Next, the same beads were re-subjected to fresh cleavage cocktail. Eluates from the first and second exposures were analyzed separately by HPLC. These results (Fig. 3f) show that indeed some residual compound remains attached to the beads following the first exposure to cleavage cocktail. Elution experiments (see below) support the notion that this observation is not due to incomplete elution of material that has been covalently cleaved. However, since the quantities involved are small relative to the first exposure ($\sim10\%$), and owing to the intrinsic hazards of working with HF solutions, we elected not to employ multiple exposures as part of our general method-

ology. Rather, we use a single 300 min exposure to a cocktail of HF/py+py in THF as our optimal protocol for cleaving compounds from trialkylsilyl linker **1**. In some cases, particularly with secondary and phenolic silyl ethers, adjustments to this protocol are warranted. In general, our data reflect these trends and may be used as guidelines in formatting libraries of specific compounds.

2.3. Optimization of compound elution conditions

Following evaporation of the by-products of quenched cleavage reactions, library compounds must be eluted from the PS support. Even less work has been directed at elution studies than at compound cleavage, particularly for large PS beads. Indeed, the knowledge based on compound elution is largely either borrowed from the long history of solid-phase peptide synthesis or inferred from studies of the physicochemical properties of different resins [23]. Our earlier attempts to elute compounds efficiently from large PS beads were less than satisfactory [9], most likely due to the use of dimethylsulfoxide (DMSO) as an elution solvent. To address compound elution from large PS beads systematically, we performed a series of optimization experiments analogous to those described above, using **3** as the model substrate.

We chose five solvents to compare directly their ability to elute cleaved compounds from the PS matrix: dichloromethane (CH_2Cl_2), CH_3CN, dimethylformamide (DMF), DMSO, and THF. Individual beads from resin batch **3** were exposed to our 'best practices' cleavage cocktail for 180 min, reactions quenched with TMSOMe, and the solvent and volatile by-products removed by evaporation. Compound **6** was then eluted from each bead by two successive 20 μl washes in one of the solvents, which were pooled for HPLC analysis. These results demonstrate that CH_3CN and DMF are superior to the other three solvents at eluting **6** (Fig. 4a). Notably, DMSO performed half as well as either CH_3CN or DMF, while THF gave yields less than 10% those of CH_3CN or DMF. As there is no significant difference in yield between CH_3CN- and DMF-based elution, we suggest that either solvent might be used for efficient elution from large PS beads.

To test whether multiple iterations of elution would serve more effectively to extract compound **6** from the resin, we exposed individual beads from batch **3** to HF/py+py cocktail for 180 min, quenched, and evaporated. Next, we subjected each bead to six successive rounds of elution with 20 μl CH_3CN. Rather than pooling these eluates as before, we kept each 20 μl aliquot separate in order to compare the yield in the first elution with that in each subsequent elution. As we expected, most of the compound (59%) was released during the first elution iteration (Fig. 4b). However, a significant portion of **6** emerged during the second (28%) and later (9% total) elutions steps, suggesting that pooling multiple elutions from large PS beads is effective in generating stock solutions containing large quantities of compound. As the iterations proceed, the signal from successive elution steps drops below the threshold for HPLC detection. It is important to realize that this places an upper limit on any claim we make about elution efficiency. When these data are weighted by their individual signal-to-noise ratios, and the results plotted as a cumulative percent elution across all six iterations (Fig. 4c), it is evident that maximal elution (96.2%) is accomplished after four elution iterations.

Finally, we explored whether increasing the length of time spent in each cycle of CH_3CN elution would improve the elution of **6**. For comparison, all previous experiments involved the addition and immediate withdrawal of solvent, such that the soak time in elution solvent was < 3 s. Here, replicate beads from batch **3** were independently cleaved as before, and each bead was subjected to four 20 μl elution cycles in CH_3CN for 1 min, 3 min, or 10 min. Surprisingly, this optimization provided the largest overall improvement in absolute yield. With 1 min soak times in each cycle, an average of 60% more of **6** was removed from the bead than without waiting. On average, 109% more of **6** was eluted with 10 min soak times. Similar experiments, in which resin **4** or **5** was subjected to cleavage and then eluted with 10 min soak times, likewise gave

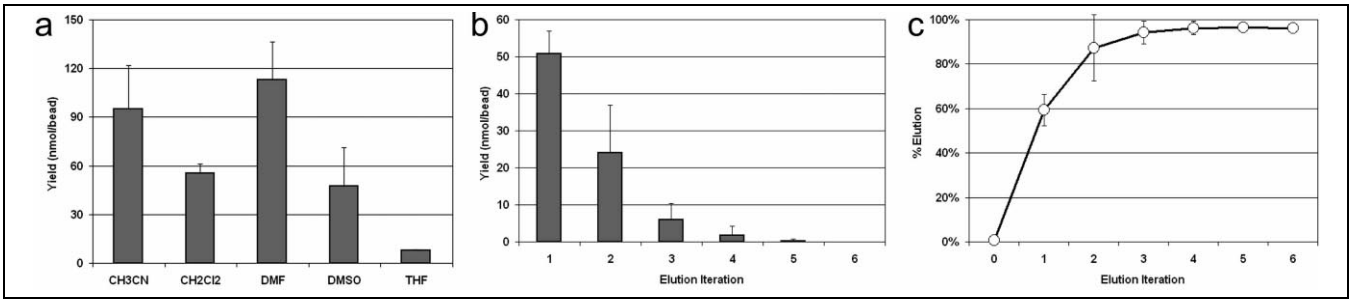

Fig. 4. 'Best practices' compound elution. a: Selection of elution solvent. Resin **3** was treated for 180 min with HF/py+py to generate **6**. After quenching and evaporation, beads were subjected to two 20 μl washes in the indicated solvent. Data are plotted as the mean ± S.D. for six independent reactions. b, c: Multiple elution washes. Resin **3** was treated for 180 min with HF/py+py to generate **6**. After quenching and evaporation, beads were subjected to six 20 μl washes in CH_3CN, which were analyzed separately. In (b), data are plotted as the mean ± S.D. nmol/bead for six independent reactions. In (c), data are plotted as the mean ± S.D. cumulative % elution, corrected for per-bead signal-to-noise ratio (max = 96.2%), for six independent reactions.

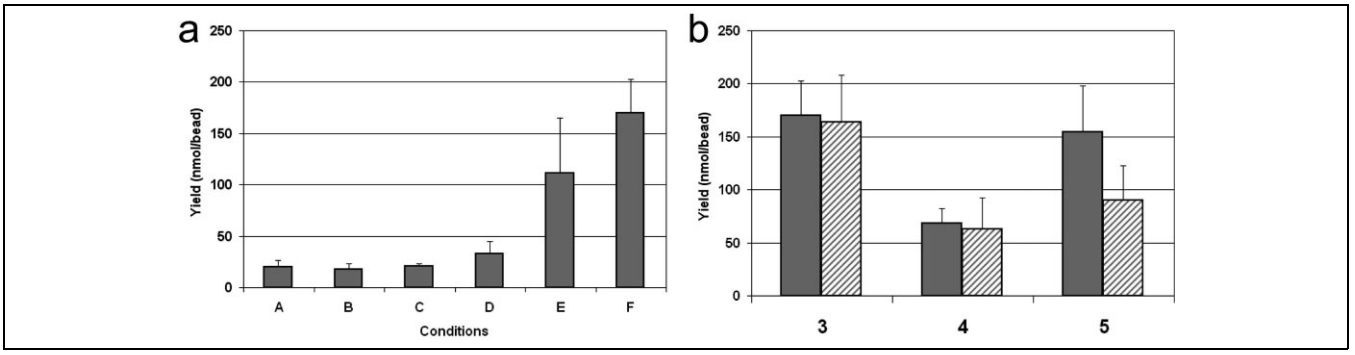

Fig. 5. Summary of optimization and robotic implementation. a: Summary of optimization. Data are plotted as the mean ± S.D. for at least six independent instances of cleavage (in THF solution) and elution (into CH_3CN) of **6** from resin **3**. Conditions: A, 30 min 5% HF/py, 2×3 s washes; B, 30 min 5% HF/py+20% pyridine, 2×3 s washes; C, 30 min 5% HF/py+5% pyridine, 2×3 s washes; D, 180 min 5% HF/py+5% pyridine, 2×3 s washes; E, 300 min 5% HF/py+5% pyridine, 4×3 s washes; F, 180 min 5% HF/py+5% pyridine, 4×10 min washes. b: Robotic implementation. Resins **3**–**5** were treated under optimized 'best practices' conditions to generate **6**–**8**. For manual experiments (solid bars), data are plotted as the mean ± S.D. for five independent reactions. For robotic experiments (hatched bars), data are plotted as the mean ± S.D. for 10 independent reactions.

increased average yields of **7** (177%) and **8** (52%), respectively. In summary, optimization of cleavage and elution parameters resulted not only in the identification of a general protocol for releasing compounds from trialkylsilyl linker **1**, but also in a several-fold improvement of total compound yield. Furthermore, we have uncovered several trends that will aid in formatting libraries that are linked to the solid phase through secondary or phenolic silyl ether linkages. Our optimization procedure proceeded iteratively, such that optimal parameters found in initial experiments were used in subsequent experiments. Consequently, we can readily plot the increase in overall yield across the series of manual optimization experiments (Fig. 5a).

2.4. Robotic implementation of cleavage and elution

In order to implement the cleavage and elution protocol robotically, a number of additional challenges had to be surmounted. Individual beads from a diversity-oriented synthesis first must be arrayed, one bead per well, into 384-well microtiter plates. Next, each bead must successively be exposed to the cleavage cocktail, the quenching reagent, and multiple instances of an elution solvent (CH_3CN or DMF). Throughout this procedure, the positional integrity of the beads and corresponding stock solutions must strictly be maintained. In other words, each bead must remain associated with exactly one well in both the cleavage plate and the 'mother plate' into which successive elutions are pooled.

Our solution to bead arraying is conceptually straightforward. We have optimized the use of a bead arrayer that immobilizes 384 beads in an equal number of depressions (Fig. 6a). The arrayer is designed for connection to both a standard vacuum line and a standard nitrogen line. Beads are trapped by application of vacuum to the apparatus, so that excess beads (Fig. 6b) can be brushed away easily and recovered, leaving exactly 384 beads on the arrayer in a regular 24×16 matrix (Fig. 6c). To deposit individual beads into the wells of a microtiter plate, the arrayer is inverted onto the plate, such that one bead is suspended over each well of the plate, and the vacuum relieved by pressurization with nitrogen. Beads are thus pushed out of the depressions and down into the wells of the microtiter

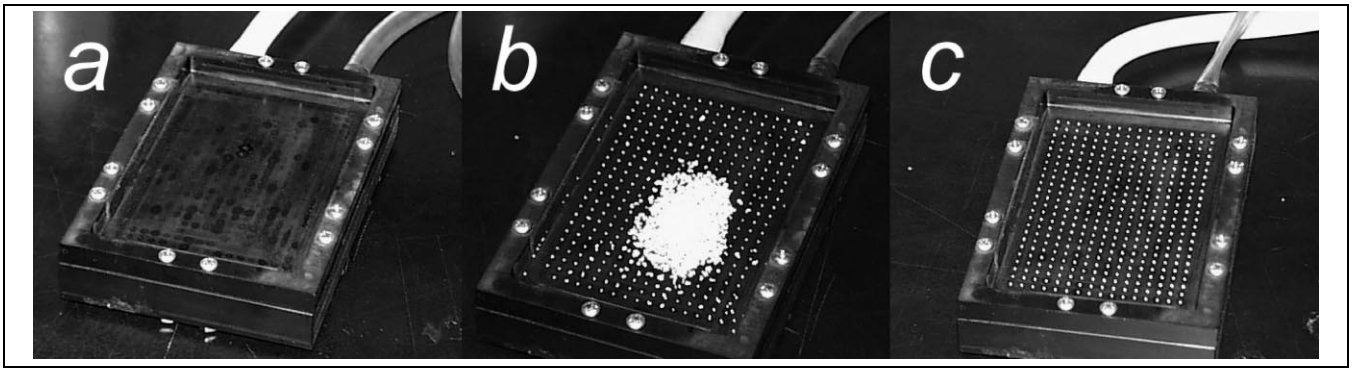

Fig. 6. Operation of bead arrayer. The bead arrayer is (a) attached to standard nitrogen and vacuum lines. Vacuum is applied and beads are (b) decanted onto the platform for entrainment by 384 depressions. Excess beads are recovered, leaving (c) a regular array of 384 beads with identical spacing to a standard 384-well microtiter plate.

plate. In practice, we have found that pre-wetting the wells with a small portion of THF or CH_3CN (10 μl/well, delivered by a bench-top plate filler) dramatically simplifies this procedure. Dry beads tend to cling to the walls or lip of a well due to static electricity. Pre-wetting the wells with solvent serves to trap the bead at the bottom, which greatly eases subsequent plate handling. Furthermore, when done just prior to adding cleavage cocktail, a portion of THF serves to pre-swell the resin, which modestly enhances the rate of the cleavage reaction (data not shown).

HF/py cannot be dispensed using conventional liquid handling robots due to the highly corrosive nature of this reagent. At issue are both the capture of toxic vapors, to minimize hazards to both people and equipment, and the composition of materials that come into direct contact with HF/py solutions. The first of these issues was addressed by encasing our robotic library formatting station in a specially designed ductless fume hood equipped with a recirculating air filtration system. In particular, this hood provides adequate ventilation of the station, as well as protective shields that isolate the operator from the robotics during operation. To dispense HF/py safely, we used a ceramic pump system coupled to both a rectilinear dispenser head (fitted with HF-resistant tubing) and a standard plate-stacker to deliver microtiter plates to the dispenser platform. We use the same instrumentation to deliver TMSOMe to cleavage reactions, which has the added benefit of quenching the pumps and tubing, converting any residual HF into non-corrosive volatile byproducts. To evaporate quenched cleavage reactions, we use a temperature-controlled vacuum centrifuge equipped with microtiter plate adapters. To elute compounds from the beads, we use a syringe-array liquid handling robot coupled to another plate-stacker. This combination is able to effect compound elution and pooling of stock solutions into mother plates at a rate of 4–6 plates/h.

To validate the portability of our optimized cleavage and elution protocol to this robotics environment, we arrayed 384 beads from each of batches **3**–**5** into three microtiter plates using the bead arrayer. Each plate was subjected to treatment with our HF/py+py cocktail, delivered robotically, followed by TMSOMe after 300 min (or ethoxytrimethylsilane; S.B. Park and S.L.S., unpublished results). Following evaporation of quenched reactions, each bead was subjected to four 10 min elution iterations, each in 20 μl CH_3CN, which were pooled into fresh 384-well 'mother plates'. Aliquots from 10 wells of each plate were subjected to HPLC analysis as described previously. This experiment illustrates the success of robotic implementation of our strategy using model compounds (Fig. 5b). For each of **6**–**8**, the robotic cleavage and elution protocol adequately reproduces the same experiments carried out manually. In all cases, the absolute yield of **6**–**8** is greater than our goal of 50 nmol/bead.

2.5. *Cleavage and elution of a diversity set of dihydropyrancarboxamides*

To prove useful, the optimized robotic cleavage and elution protocol must be able to deliver actual library members from diversity-oriented syntheses into chemical genetic assays. To validate this approach, we used an encoded, split-pool library of 4320 dihydropyrancarboxamides (**10**), whose development and synthesis are described in detail elsewhere [27]. Briefly, this library consists of three diversity-generating steps, the first two of which were encoded with chloroaromatic tags as described in the preceding paper in this issue [15]. As the final diversity-generating step was not chemically encoded, we acquired this library as 54 separate portions of dry resin (**9**) totaling three theoretical copies of 4320 stereochemically and structurally distinct compounds (**10**). We first exposed 324 individual beads, six from each of the 54 separate portions of **9**, to our manual 'best practices' cleavage and elution conditions (Fig. 7) in a single microtiter plate. In this case, compounds were eluted directly into DMF to prepare a diversity plate of stock solutions (plate 0) amenable to small molecule printing.

Glass microscope slides were activated for covalent attachment of alcohols, and compounds (**10**) from the 320 stock solutions were printed as described previously [3]. To test the availability of **10** to a protein-binding assay, we probed the small molecule microarray with purified

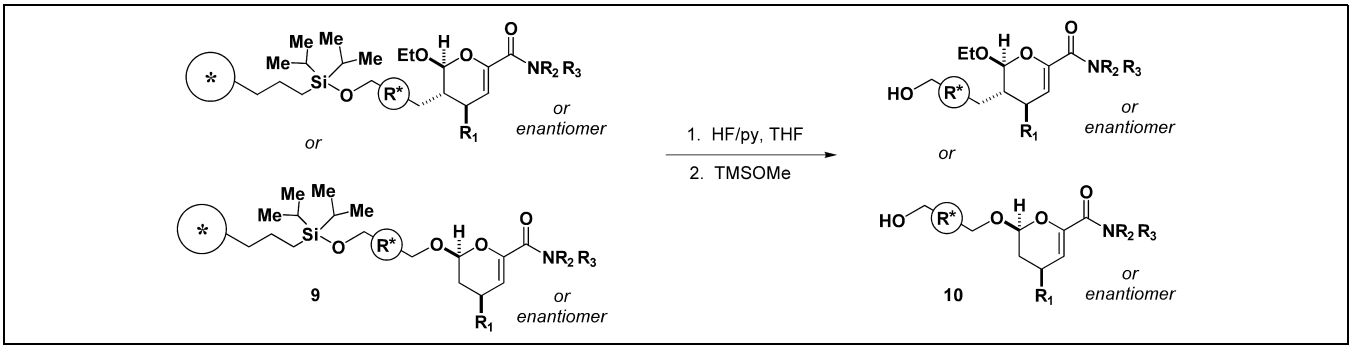

Fig. 7. Cleavage scheme for dihydropyrancarboxamides. Individual beads from separate portions of resin **9** were treated under optimized 'best practices' conditions to generate stock solutions of individual members of **10**.

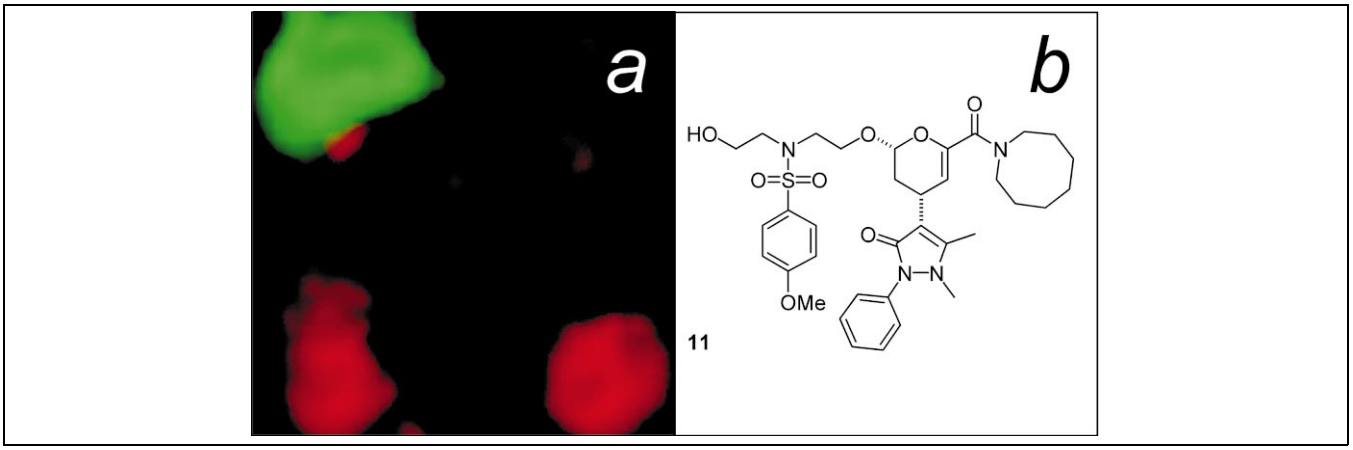

Fig. 8. Representative reverse chemical genetic assay. A small molecule microarray containing members of **10** was probed with purified Cy5-labeled $(His)_6$-FKBP12. a: Fluorescence intensity at duplicate spots (false-colored red) containing a 'hit' is shown compared to a rhodamine control spot (false-colored green). b: The structure of the 'hit' (**11**) was determined by bead decoding and confirmed by LC/MS.

Cy5-labeled $(His)_6$-FKBP12 [2]. As a positive control for protein–ligand interaction, AP1497 [24,25] was included on the slide by adding it in DMF solution to an empty well of the stock plate. Following incubation, the slide was washed and scanned for the presence of a Cy5 fluorescence signal [2], which appeared both at the AP1497 control spots (data not shown) and at spots corresponding to a member of **10** (Fig. 8a). The bead corresponding to the novel FKBP12-binding entity was subjected to the optimized bead decoding protocol described in the preceding paper in this issue [15]. Using this procedure, we were able unambiguously to determine the structure (Fig. 8b) of this 'hit' (**11**) in a protein-binding assay, as was subsequently confirmed by tandem liquid chromatography/mass spectroscopy (LC/MS).

2.6. *Formatting and assaying of representative dihydropyrancarboxamides*

To apply the robotic process to a fraction of resin **9**, we arrayed 128 beads from each of three separate portions of **9** into a single 384-well microtiter plate. These beads were subjected to robotic cleavage and CH_3CN elution as described earlier to prepare a 'mother plate' (plate 1) containing 384 members of **10**. Subsequently, the 'mother plate' was mapped into six 'daughter plates' by volumetric transfer using the syringe-array robot. 'Daughter plates' were prepared for cell-based assays [1,8] (50% of stock solution), HPLC analysis (25%), LC/MS analysis (10%), small molecule printing [2,3] ($2\times5\%$), and stock solution decoding (5%). In each case, the CH_3CN solution was evaporated following volumetric transfer so that each copy could be resuspended in the solvent most appropriate to its use. In particular, DMSO was used to resuspend the 'daughter plate' for cell-based assays and DMF was used to resuspend the 'daughter plate' for small molecule printing. The plate containing the beads was also stored, but due to the success of stock solution decoding [28], and the difficulties associated with maintaining positional integrity within plates of beads, formatting a 'daughter plate' explicitly destined for structure determination has become the standard in our library realization process.

Both plates of stock solutions (**10**) were used in phenotypic assays. In particular, we exposed living human A549 lung carcinoma cells to 708 (324+384) stock solutions under two different assay conditions. These experiments were performed with a hand-held pin-transfer tool, though our complete technology platform includes a pin-transfer robot capable of mapping into multiple microtiter plates. In general, cultured cells exposed to 5-bromodeoxyuridine (BrdU) will incorporate this base analog into their DNA when actively dividing, and this incorporation can be detected by cytoblot assay using antibodies directed against BrdU [8]. First, to determine if any stock solution of **10** inhibits BrdU incorporation, we transferred $\sim$100 nl of each stock solution into individual assay wells containing A549 cells actively growing in the presence of 1% fetal bovine serum. Second, we exposed A549 cells to $\sim$100 nl of each stock solution, and simultaneously challenged the cells with 100 μM genistein, a broad-spectrum protein tyrosine kinase inhibitor [26]. Under the latter conditions, BrdU incorporation, again judged by cytoblot assay [8], is impaired. Thus, 'hits' in the former assay are detected as a loss of signal in a high-signal array (Fig. 9a), while 'hits' in the latter assay are detected as a gain of signal in a low-signal array (Fig. 9b). The latter assay is referred to as a genistein suppressor screen, as we are seeking a member of **10** that can suppress the ability of genistein to inhibit BrdU incorporation.

For each of these assays, aliquots from each of the two plates (**10**) were exposed to cells in duplicate to ensure the fidelity of the results. Compounds were scored as 'hits' only if they scored strongly in both replicates of a given experiment. From plate 0, 11 compounds scored as inhibitors of BrdU incorporation, while 10 compounds scored as suppressors of the action of genistein. From plate 1, 12

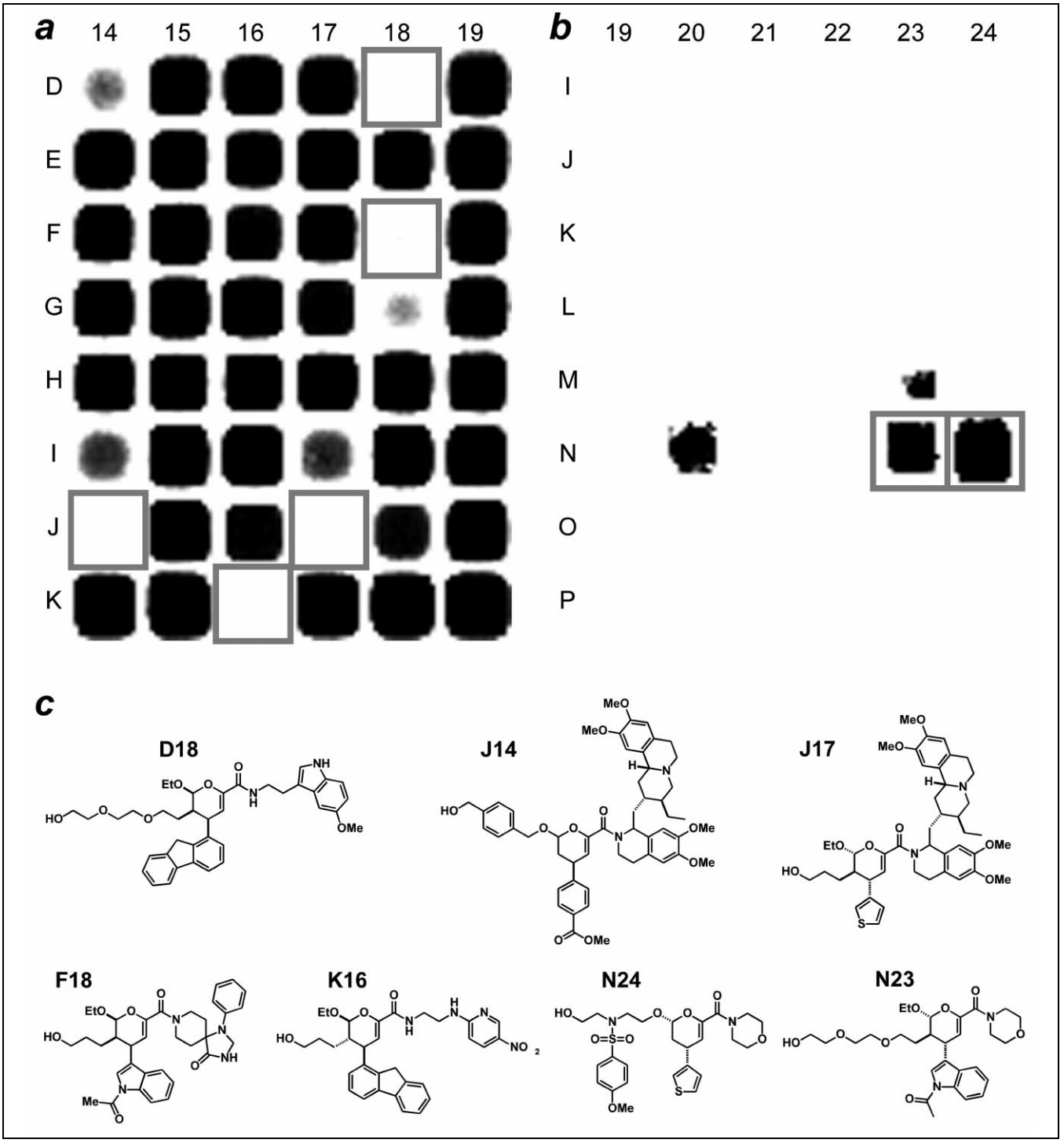

Fig. 9. Representative forward chemical genetic assays. Human A549 cells were exposed in duplicate to stock solutions of **10**. 'Hits' (gray boxes) are those wells that scored in both replicates of a given experiment. Data from 48 representative wells are shown as multiplicative overlays of cytoblot results from (a) a BrdU incorporation assay, and (b) a genistein suppressor screen. c: Structures of representative 'hits'. Beads or stock solutions corresponding to 'hits' in cytoblot assays were exposed to the optimized decoding protocol described in the preceding paper in this issue. Compounds are labeled by well position in the assay plates.

compounds scored as inhibitors of BrdU incorporation, while nine compounds scored as suppressors of the action of genistein. It is interesting that roughly the same number of first-pass 'hits' were identified on each plate, despite the difference in diversity between the two collections. This finding may reflect the fact that assay results were tabulated by visual scoring of photographic film, but is not limited to such detection methods. Conversely, in the case of an FKBP12-binding assay using microarrayed compounds, plate 1 produced no 'hits' (data not shown).

To ensure that we can obtain exact structural information on the 'hits' found in these experiments, we performed either bead decoding [15] or stock solution decoding [28] on all 42 compounds scoring as positive in either assay. Decoding results were compared with LC/MS results for each sample to verify that a compound of the correct mass was present. In all but nine cases, LC traces revealed a single clean peak, and for each of the 42 'hits', a parent ion or fragment matching the proposed structure was observed by MS. Thus, we were able to decode and confirm the structure (Fig. 9c) of each 'hit' detected in either the BrdU or the genistein suppressor cytoblot assay.

From a statistical perspective, the library of dihydropyrancarboxamides (**9**) was fully encoded, either chemically using chloroaromatic tags (first two diversity-generating steps), or positionally by inclusion into one of 54 pools of resin (third diversity-generating step). Our collection of decoded 'hits' was analyzed to assign statistical significance to a process of 'codon' selection, by a given assay, of particular encoded events (or combinations of encoded events) during the chemical history of the library. The formal details of this analytical process will be reported once applied to the entire collection of 4320 dihydropyrancarboxamides (**9**). One immediate consequence is that a consensus set of structures corresponding to a particular assay activity need not be limited to individual structures that scored as 'hits' in the assay. For example, if two codons corresponding to building blocks from two different diversity-generating steps were each strongly selected by a given assay, one might predict that a compound incorporating both moieties would yield higher potency in that assay. In the absence of additional information, we would predict such a consensus structure even if the exact compound in question was not present in the initial screen. Alternatively, if the assay in question selected against this particular combination of codons, we would uncover this 'forbidden' combination, even if each codon alone was frequently observed among structures scoring as 'hits'. Traditionally, structure–activity relationships are determined by processes ranging from an intuitive viewing of 'hit' structures to a comparison of 'hits' on the basis of existing quantitative molecular descriptors (each based on some arbitrary metric). Our analysis introduces a novel approach, whereby we require no structural information in advance of defining significant biological activity. Rather, we allow the biological system under study to dictate the requirements for its activity. Such analysis illustrates the power of annotation screens to inform chemistry, through the technology platform, in ways that can influence planning steps in future diversity-oriented syntheses.

3. Significance

We have outlined the second phase of development of a technology platform aimed at advancing chemical genetics. The platform consists of, in part, an optimized procedure for compound cleavage and elution from large PS beads, a novel bead arraying method, and robotic implementation of library formatting, the process by which small molecules from diversity-oriented syntheses are made accessible to chemical genetic assays. We validated this approach by successfully synthesizing, encoding, and formatting a split-pool library of dihydropyrancarboxamides (**9**). It is important to note that optimization of the library formatting process occurred independently of the development of chemistry required to synthesize the library. Rather, optimization of the formatting process used generic model compounds to establish parameters, while formatting the split-pool library used the output of the optimization as a general, or 'best practices', method for library realization.

By exposing each member of a diversity-oriented synthesis to multiple phenotypic and proteomic assays, we can annotate each compound in the collection in a way that is complementary to other methods of small molecule characterization, such as MS and NMR. Statistical analysis of the biological performance of an encoded collection of small molecules allows us to inform further synthetic efforts (e.g. scaled synthesis of subset libraries based on primary screening data) in ways not necessarily available by traditional structure–activity analyses. Annotation screening is a term we use to describe the generation of multiple datasets by comprehensive screening of such libraries over a range of biological outcomes. The analysis of data resulting from annotation screening comprises both the challenge and the promise of chemical genetic research.

4. Materials and methods

4.1. Model resin preparation

Compounds **6** (2-naphthaleneethanol), **7** (α-methyl-2-naphthalenemethanol), and **8** (2-naphthol) were obtained commercially (Sigma-Aldrich) and dried azeotropically prior to the loading reaction. Resin **1** was a generous gift of Max Narovlyansky and Dr. John A. Tallarico, and contains ~200 nmol Si/bead calculated based on elemental analysis, assuming that 550 μm is the average bead size in a population of beads pre-sized at 500–600 μm. Loading reactions were performed in fritted polypropylene PD-10 columns (Amersham Pharmacia Biotech) and agitated by rocking on a Labquake™ (Barnstead Thermolyne) shaker. Resin samples were washed on a Vac-Man® vacuum manifold (Promega) fitted with nylon stopcocks (Bio-Rad). HPLC-grade reaction solvents (J.T. Baker) were purified by passage through two solvent columns prior to use. Et_3N and 2,6-lutidene were distilled over calcium hydride. In loading reactions, bromostyrene-copolymerized beads were added to a PD-10 (Amersham Pharmacia Biotech) column, which was capped with a septum and plastic stopcock and flushed with Ar. After swelling with

CH_2Cl_2 (10 ml), a 2.5% (v/v) solution of TMSCl in CH_2Cl_2 was added. The beads were suspended for 15 min and filtered with Ar pressure. The beads were washed with CH_2Cl_2 (3×2 min), then suspended in a solution of TfOH (6 eq.) in CH_2Cl_2 for 15 min, during which time Ar was bubbled gently through the reaction via a syringe. Next, the beads were rinsed with CH_2Cl_2 (3×2 min) under Ar and suspended in CH_2Cl_2. Freshly distilled 2,6-lutidine (8 eq.) and model alcohol **6**, **7**, or **8** (3 eq.) were successively added. The tube was capped and sealed to stand for 18 h at ambient temperature, after which the beads were filtered and rinsed with CH_2Cl_2 (4×3 min) and dried under house vacuum.

4.2. Cleavage and quenching

Commercially available HF/py (Sigma-Aldrich) is approximately a 7:3 mixture of HF and pyridine, which was buffered with additional pyridine in THF solution. In manual experiments, beads were transferred individually by forceps to wells of 384-well microtiter plates (Genetix). Cleavage and quenching reagents, as well as elution solvents, were added by a P20 single-channel pipettor (Gilson). Data from ^{19}F NMR experiments were obtained at 470.169 MHz on a Varian (Varian, Inc., http://www.varianinc.com/) AS500 (nt = 128). To avoid etching of the NMR tube by HF/py solutions, samples were placed in a PTFE-FEP NMR tube liner (Wilmad-LabGlass).

4.3. HPLC quantitation

HPLC analysis was carried out using a ThermoSeparation Products (Thermo-Finnigan) instrument with a PC1000 system controller and associated software. All samples were run on a Hypersil C18 mini-pharmaceutical column (The Nest Group) using a flow rate of 3 ml/min, an 80 s gradient of 0–99.9% CH_3CN in water/0.1% trifluoroacetic acid/0.1% methanol, and diode array detection. Single peaks at 224 nm absorbance were characteristic of compounds **6** (rt = 1.54 min), **7** (rt = 1.54 min), and **8** (rt = 1.49 min). To establish boundary conditions for detection of cleaved compounds by HPLC, standard curves were determined using pure samples of **6**–**8**. Mock cleavage reactions (no HF present, but otherwise treated as described in the text) were carried out on resins **3**–**5** to determine the experimental noise for our HPLC detection method.

4.4. Robotic implementation

Before bead arraying, 384-well plates (Genetix) were pre-wetted using a Multidrop 384 (Thermo-Labsystems) to dispense solvent. HF/py solutions were delivered using an Ivek multiplex controller module with linear actuator pump module (Ivek Corporation, http://www.ivek.com/) coupled to an ADM-661 automatic dispensing system with TruPath 300 controller module (Creative Automation, http://www.creativedispensing.com/), and fully contained within a Captair ductless fume hood with recirculating air filtration system (Captair LabX, http://www.erlab-dfs.com/). Automated plate handling was carried out by Twister Universal microplate handlers (Zymark Corporation, http://www.zymark.com/). Evaporation of quenched reaction mixtures was done using a GeneVac HT4 Atlas evaporator with VC3000D vapor condenser (GeneVac Technologies, http://www.genevac.co.uk/). Elution of compounds from beads into 100 μl/well 'mother plates' (Marsh), as well as formatting of 50 μl/well 'daughter plates' (Genetix), was done with a Hydra Microdispenser 384 (Robbins Scientific Corporation, http://www.robsci.com/).

4.5. Small molecule microarrays

Small molecules were printed as described in [3], either with a microarray robot built as described by Dr. Pat O. Brown (http://cmgm.stanford.edu/pbrown/mguide/), or with an Omni-Grid™ multi-axis robot (GeneMachines, http://www.genemachines.com/). $(His)_6$-FKBP12 was purified to homogeneity as described in [2]. Cy5-labeled protein was prepared using FluoroLink™ monofunctional reactive dye (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Fluorescence detection of binding events was monitored using an ArrayWoRx biochip reader (Applied Precision, http://www.api.com/).

4.6. Cell-based assays

Transfer of stock solutions of **10** into assay plates (Nunc) was done using a VP386 384-pin MultiBlot™ replicator (V&P Scientific, http://www.vp-scientific.com/). Cell culture methods and the BrdU assay protocol were carried out exactly as described in [8]. Detection of assay results was carried out using X-oMAT AR film (Kodak), and multiplicative overlays of digitally scanned replicate films were prepared using Photoshop 5.0 (Adobe Systems).

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