Journal of Cellular Biochemistry Supplement 37:7–12 (2001)

New Technologies for Chemical Genetics

Leslie A. Walling, Noël R. Peters, Edward J. Horn, and Randall W. King*

Institute of Chemistry and Cell Biology, Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115

Abstract Chemical genetics, in which small molecules are used in lieu of mutations to study biological processes, requires large and diverse chemical libraries to specifically perturb different biological pathways. Here we describe a suite of technologies that enable chemical libraries prepared by split-pool solid phase synthesis to be screened in a diverse range of chemical genetic assays. Compounds are synthesized on 500 micron high-capacity polystyrene beads, and arrayed into individual wells of 384-well plates using a hand-held bead arrayer. Compounds are cleaved from synthesis beads using a chemically-resistant ceramic dispensing system, producing individual stock solutions of single compounds. Nanoliter volumes of these solutions are then transferred into assay plates using an array of stainless steel pins mounted on a robotic arm. We have designed reusable 1536- and 6144-well assay plates made of silicone rubber that can be cast in the laboratory and filled by hand. This integrated technology platform enables hundreds of biological assays to be performed from the product of a single synthesis bead, enabling the results of different chemical genetic experiments to be directly compared. J. Cell. Biochem. Suppl. 37: 7–12, 2001.

Key words: chemical genetics; combinatorial chemistry; bead arrayer; high-throughput screening; compound transfer; assay plates; automation

Chemical genetics is an emerging approach in which chemical libraries are used to perturb biological systems to identify proteins that participate in biological pathways [Mitchison, 1994; Crews and Splittgerber, 1999]. To be successful, the approach requires that diverse chemical libraries be screened in a broad range of biological assays [Mayer et al., 1999; Peterson et al., 2000; Rosania et al., 2000; Degterev et al., 2001]. The development of split-pool combinatorial synthesis has made it possible to generate large chemical libraries in academic laboratories. New concepts in diversity-oriented chemical synthesis [Schreiber, 2000] have enabled the creation of chemical libraries with structural features reminiscent of natural products [Tan et al., 1998; Pelish et al., 2001]. However, libraries generated by this method create several challenges for screening. The identity of each compound must be recorded by encoding procedures [Ohlmeyer et al., 1993] or determined by direct chemical analysis. Compounds must be released from beads to perform cell-based assays. Finally, the limited amount of compound produced by each bead has made it difficult to perform more than a few assays.

To address these challenges, we have developed a suite of technologies that process synthesis beads into an array of individual stock solutions that can be efficiently screened in a broad range of assays. A summary of the approach is shown in Figure 1. Following completion of a synthesis, high-capacity polystyrene beads, carrying 150 nanomoles of compound, are arrayed into individual wells of 384-well plates. Compounds are cleaved from the beads using a ceramic dispensing system, and resuspended in 15 μl of DMSO to produce individual stock solutions of approximately 10 mM concentration. A robot-mounted stainless steel pin array then transfers 10–100 nl of the stock solution into assay plates. To conserve compound and reagent use, we have developed reusable 1536- and 6144-well assay plates that are cast from silicone rubber. These

Grant sponsor: National Cancer Institute; Grant number: CA78048; Grant sponsor: Merck & Co.; Grant sponsor: Merck KGaA.

Leslie A. Walling's present address is Amgen Inc., M/S 29-1-A, One Amgen Drive, Thousand Oaks, CA 91320.

*Correspondence to: Randall W. King, Department of Cell Biology, Harvard Medical School, 250 Longwood Ave, SGM 604 Boston, MA 02115.

E-mail: randy_king@hms.harvard.edu

Received 19 September 2001; Accepted 19 September 2001

DOI 10.1002/jcb.10075

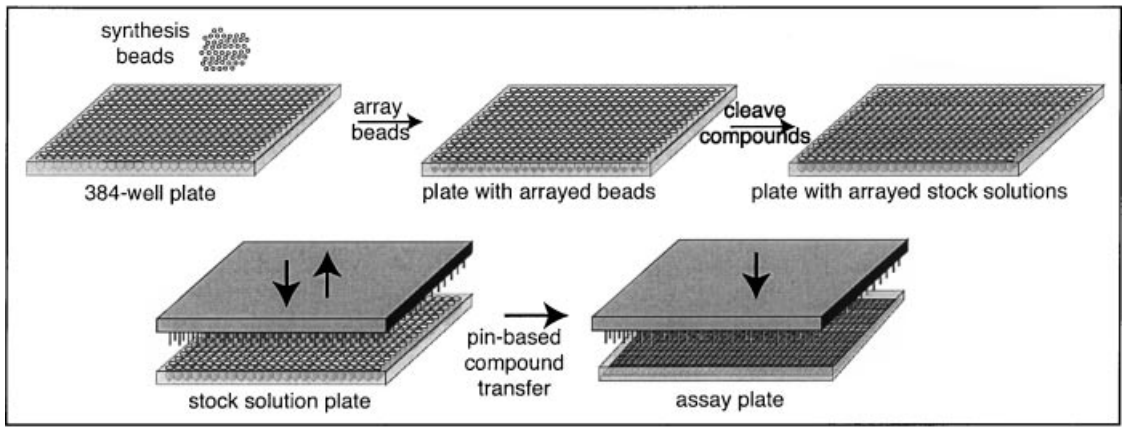

Fig. 1. Summary of the approach used to generate and screen arrayed split-pool chemical libraries. Beads are first arrayed into individual wells of 384-well polypropylene plates. Compound is cleaved from the arrayed beads by delivery of cleavage solution using an automated ceramic dispensing system. Compounds are subsequently resuspended in DMSO to produce stock solutions. An array of 384 pins is then used to transfer nanoliter volumes of liquid from compound storage plates to assay plates.

technologies enable the product of a single synthesis bead to be screened in over a hundred assays. Arrayed stock solutions minimize the amount of decoding that is required, as a particular stock solution does not need to be decoded more than once. Results of different chemical genetic screens using arrayed libraries can be directly compared, providing important information regarding the selectivity of compounds, aiding the prioritization of hits for the subsequent steps of decoding and resynthesis.

Bead Arraying

To array synthesis beads, we developed a hand-held vacuum manifold that contains an array of 384 micromachined holes, each 300 microns in diameter (Fig. 2A). Dry synthesis beads are sprinkled on top of the device, and when a vacuum is applied, negative pressure traps a 500 micron bead in each hole. Excess beads are removed with a brush, producing an array of 384 beads (Fig. 2B). The device is then inverted over a 384-well polypropylene plate, and a brief pulse of positive pressure is applied, depositing a single bead in each well of the plate. Although we have used this system exclusively for arraying 500 micron polystyrene beads, the use of smaller holes would in principle enable smaller beads to be arrayed. In routine use, a plate of beads can be arrayed in few minutes by a single person. We have found that the inclusion of a small amount of solvent in the recipient plate enhances transfer efficiency and accuracy [Clemons et al., 2001]. Detailed plans and specifications for this device and the others described below may be found on our website at http://iccbweb.med.harvard.edu/king/index.html.

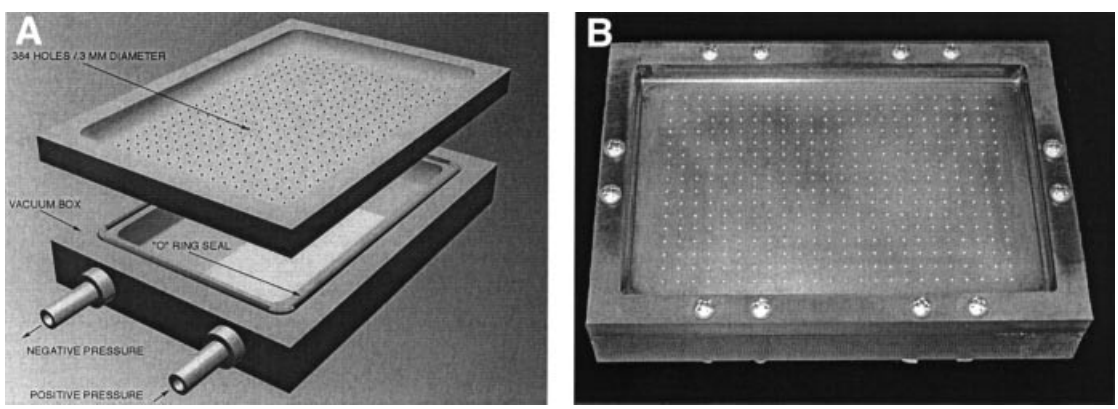

Fig. 2. Device used to array beads. **A**: Schematic of vacuum manifold device. **B**: Image of arrayer after 500 micron synthesis beads have been captured by individual holes through vacuum suction.

Compound Elution and Resuspension

Once beads are arrayed into polypropylene plates, compounds are cleaved from a silicon-based linker by addition of a dilute solution of hydrofluoric acid buffered with pyridine. Since this reagent is hazardous and reacts with glass, we integrated a chemically-resistant ceramic-based pump system (Series 100 Multiplex 8 Dispenser, IVEK Corporation, North Springfield, VT) with an automated cartesian robotic head (ADM 661; Creative Automation, Sun Valley, CA) and plate stacker (Twister, Zymark Corporation, Hopkinton, MA). This system retrieves plates from the stack, dispenses cleavage reagent to the plate, and returns the plate to the stacker to allow cleavage to occur. The plate is then returned to the instrument to dispense a quenching reagent. Volatile products are removed by centrifugal evaporation, leaving beads and compound residue behind in the wells. Compounds are then resuspended in 15 μl of DMSO solution using a 384-channel Hydra HTS workstation (Robbins Scientific, Sunnyvale, CA). Each bead has a capacity of approximately 150 nanomoles of compound [Tallarico et al., 2001], yielding a 10 mM stock solution. Detailed images of these systems are available on our website. A series of best practices have been developed for the efficient formatting and cleavage of libraries using the bead arrayer and compound cleavage system [Clemons et al., 2001].

Compound Transfer

We developed a robotic system for transferring nanoliter volumes of DMSO stock solutions from storage plates into 384-, 1536-, or 6144-well assay plates (Fig. 3). Commercially-available pipetting systems typically require microliter volumes for priming prior to dispensing even nanoliter volumes of liquid. Our goal was to develop a system that consumed only the amount of compound required for an assay, ranging from 5–100 nl of liquid. The system consists of a series of interchangeable stainless-steel pin arrays (V&P Scientific, San Diego, CA) mounted on the arm of a large cartesian robot (Seiko 3106 D-TRAN, Seiko Instruments, Torrance, CA). Compound is transferred via adhesion of liquid to the pin surface or by capillary action within a groove at the tip of the pin. By varying the shape or diameter of the pin, it is possible to transfer volumes ranging from 5 to 100 nl. The robot deck contains positions for 25 compound plates and 25 assay plates, and a system for washing the pin array between each round of transfer, eliminating the need for disposable tips or pins (Fig. 3D). The robot head is equipped with suction cups that rotate down to move plate lids (Fig. 3C), and rotate out of the way during the compound transfer step (Fig. 3B). A set of 25 plates can be processed in about 15 min, depending on conditions used for washing the pin array. Compound transfer is reproducible, with a coefficient of variation of approximately 10% from pin to pin. We have used the system to successfully deliver compound into 384-, 1536-, and 6144-well plates.

High Density Assay Plates

To increase throughput and conserve compound and reagents during screening, we have developed reusable 1536-well assay plates that

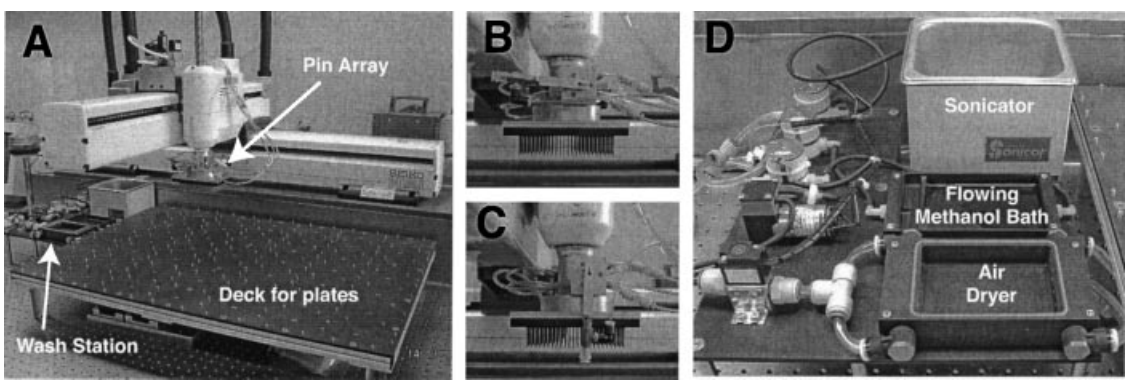

Fig. 3. Pin-based compound transfer system. **A**: Picture of robotic system consisting of a 4-axis cartesian robot, pin array, deck for plates, and wash station. **B**: Robot head containing mounted pin array. **C**: Suction cups have been rotated downward in preparation for lid removal. **D**: Wash station consisting of sonicator, flowing methanol bath, and air dryer.

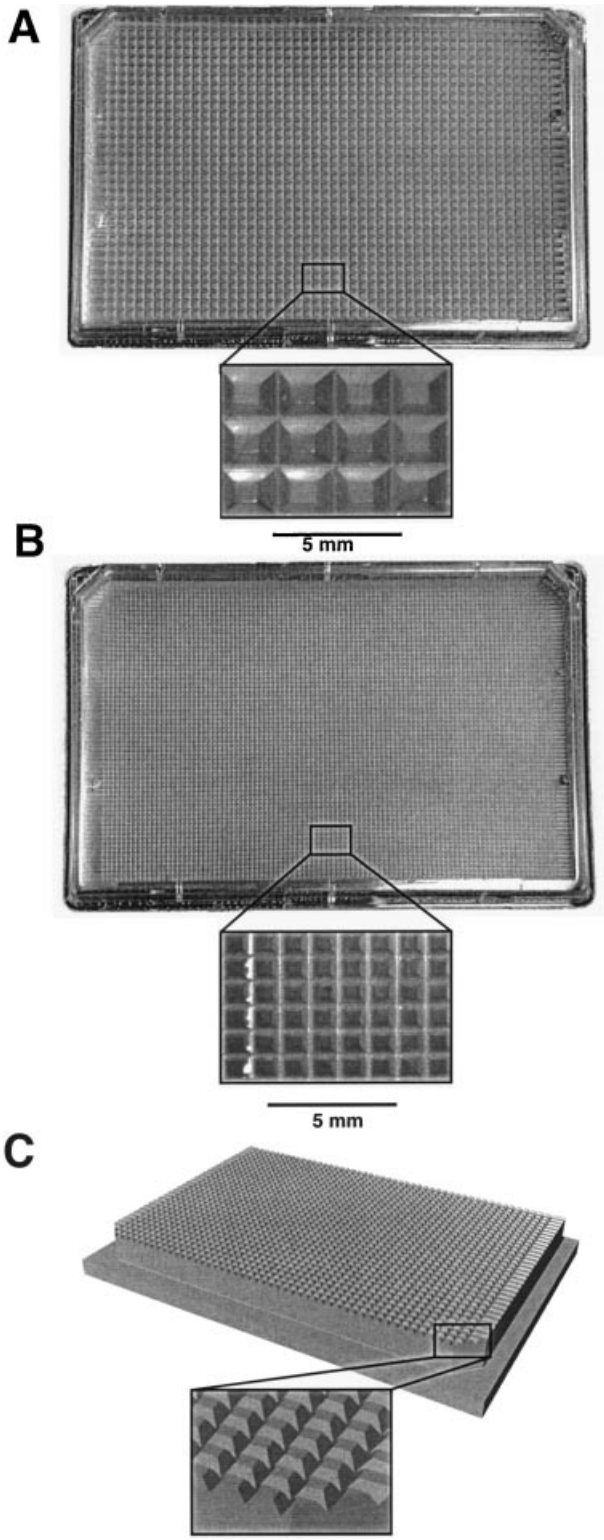

Fig. 4. High density silicone rubber assay plates and mold. **A**: 1536-well plate and enlarged view of wells. **B**: 6144-well plate and enlarged view of wells. **C**: Schematic of mold used for casting 1536-well plate.

hold 2 μl of liquid per well (Fig. 4A), and 6144-well plates that hold 250 nl of liquid per well (Fig. 4B). The wells have angled walls that facilitate reagent and compound addition, with a shape similar to that reported for other high density plates [Oldenburg et al., 1998]. We adapted a previous procedure [You et al., 1997] to cast plates in the laboratory from silicone rubber (Sylgard 184 Elastomer, Dow Chemical, Midland, MI). In this case, we designed a new mold, composed of a piece of machined acrylic, in which a series of intersecting V-shaped grooves provide the pattern for the wells (Fig. 4C). The mold is shaped to fit precisely within an inexpensive commercially-available polystyrene tray (Omnitray, Nagle Nunc International, Rochester, NY) that is used for casting and holding the rubber assay plate. Plates are cast by filling the Omnitray with liquid elastomer, and pressing the acrylic mold on top of the polymer. After curing for 24 h, the plate can be removed by peeling it from the mold. It is then placed back within the Omnitray, which holds the flexible plate during screening and enables precise positioning on deck of the compound transfer robot. Lids on the Omnitrays are used to prevent sample evaporation. Detailed mold designs and casting instructions can be found on our website.

Example Assay

The compound transfer robot and silicone rubber assay plates have been used to screen 110,000 compounds in duplicate to identify novel mitotic inhibitors (N.R.P. and R.W.K., unpublished data). Cytoplasmic extracts containing a luciferase reporter protein were plated directly onto the assay plate surface using a pipette and razor blade (Fig. 5A,B). We have found that brief treatment of the plates using a glow discharge chamber (Extended Plasma Cleaner, Harrick Scientific, Ossining, NY) increases the hydrophilicity of the plate surface and enhances the spreading of liquid. Compounds were transferred robotically from four different 384-well source plates into a single 1536-well assay plate by offsetting the pin array between each round of transfer. After incubation of the extracts with compound for 1 h, a non-contact dispensing system (Pixsys 3200 Dispenser, Cartesian Technologies, Irvine, CA) was used to deliver 250 nl of luciferin reagent to each well of the assay plate. The assay plates were then imaged using a CCD-based imaging

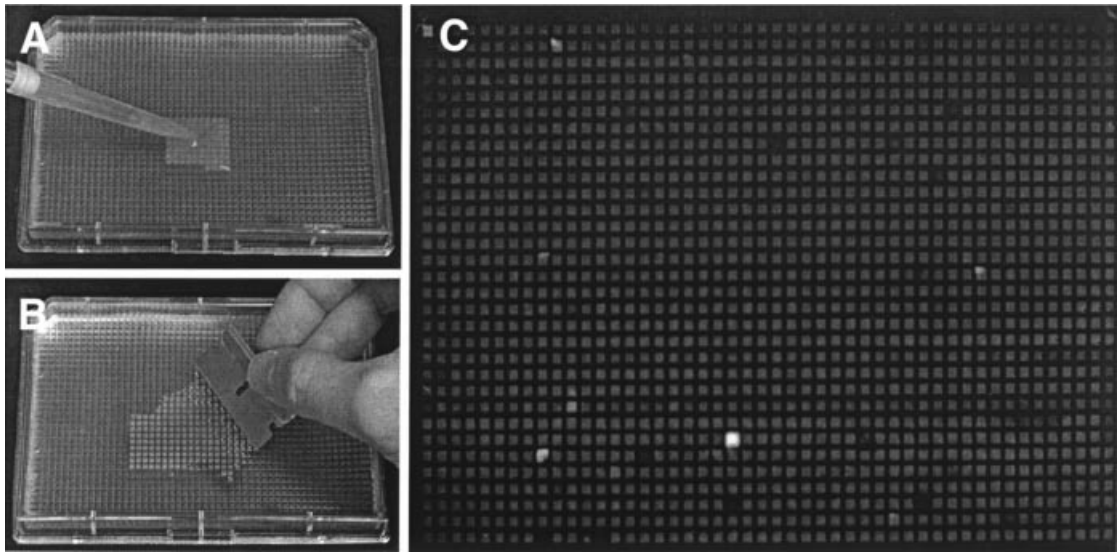

Fig. 5. Example assay. **A**: A 1536-well plate is filled by pipetting cytoplasmic extract onto the plate surface. **B**: A razor blade is used to spread liquid across the plate surface, filling all of the wells. **C**: Sample image collected with a CCD-based imaging system. The plate was filled with cytoplasmic extract containing a luciferase reporter protein. Compounds were transferred using the pin-based transfer system. Compounds that inhibit cell cycle progression result in stabilization of the luciferase reporter protein, producing a high signal. A strong hit and several weak hits can be observed.

system (Lead Seeker, Amersham Pharmacia, Piscataway, NJ). The reporter protein is degraded as the extracts progress through mitosis; small molecules that inhibit cell cycle progression result in stabilization of the reporter protein, producing a high signal. Figure 5C shows a sample image from the screen, and indicates that the pin transfer robot can deliver compounds accurately to single wells without any spill over. We processed 40 plates per day using the technologies described in this manuscript, for a throughput of 60,000 compounds per day. We washed and reused a single set of 40 assay plates four times to collect 240,000 data points for the screen.

We have found that 6144-well plates can be filled with assay reagents using a similar approach, and compounds can be successfully delivered to these plates using pin arrays. However, since 16 rounds of compound transfer are required to deliver compounds from 384-well stock plates to 6144-well assay plates, evaporation of liquid in the assay plates becomes a significant problem. By using 1536-well plates for compound storage, only four rounds of compound transfer would be required, reducing the evaporation problem. We are currently evaluating the feasibility of using 1536-well polypropylene plates for compound storage.

CONCLUSIONS

Combinatorial chemical libraries can be synthesized using a variety of approaches. Parallel synthesis enables large of amounts of each compound to be produced, and does not require the use of solid-phase synthesis resins. However, each compound must be synthesized through an independent set of reactions, limiting the number of compounds that can be produced. Directed sorting, in which solid-phase synthesis resin is packed into tagged carriers, combines the efficiency of split-pool synthesis with tracking procedures that unambiguously identify each compound. This technology can be used to synthesize libraries of over 10,000 compounds [Herpin et al., 2000], but requires large amounts of chemical reagents, making the synthesis of each library too expensive to be practical in an academic setting. Split-pool combinatorial synthesis, using high-capacity synthesis beads, enables very large libraries (over 100,000 compounds) to be synthesized in an academic setting for a reasonable cost. The amount of compound produced is sufficient to perform over a hundred assays, given the availability of screening technologies that can use the compound efficiently.

Our goal was to develop a flexible, low-cost system that would enable screening of split-pool

combinatorial libraries in a diverse range of biological assays. Although technologies exist for screening small amounts of compound released from beads [Dunn et al., 2000], these methods typically allow just a few assays to be performed from the product of a single bead. By arraying high-capacity synthesis beads [Tallarico et al., 2001], our approach enables the results of hundreds of chemical genetic experiments to be directly compared. The activity of a single compound can be determined in a broad range of chemical genetic assays, enabling investigators to identify compounds that are specific to a particular biological pathway. An important aspect of this approach is that the specificity of a compound can be assessed prior to elucidation of the structure or resynthesis of the compound. This feature is especially important when performing broad screens for ligands that affect cellular processes or pathways, as these screens typically produce large numbers of hits.

REFERENCES

Crews CM, Splittgerber U. 1999. Chemical genetics: Exploring and controlling cellular processes with chemical probes. Trends Biochem Sci 24:317–320.

Clemons PA, Koehler AN, Wagner BK, Sprigings TG, Spring DR, King RW, Schreiber SL, Foley MA. 2001. A one-bead, one-stock solution approach to chemical genetics: Part 2. Chem Biol (in press).

Degterev A, Lugovskoy A, Cardone M, Mulley B, Wagner G, Mitchison T, Yuan J. 2001. Identification of small-molecule inhibitors of interaction between the BH3 domain and Bcl-xL. Nat Cell Biol 3:173–182.

Dunn D, Orlowski M, McCoy P, Gastgeb F, Appell K, Ozgur L, Webb M, Burbaum J. 2000. Ultra-high throughput screen of two-million-member combinatorial compound collection in a miniaturized, 1536-well assay format. J Biomol Screen 5:177–188.

Herpin TF, Van Kirk KG, Salvino JM, Yu ST, Labaudiniere RF. 2000. Synthesis of a 10,000 member 1,5-benzodiazepine-2-one library by the directed sorting method. J Comb Chem 2:513–521.

Mayer TU, Kapoor TM, Haggarty SJ, King RW, Schreiber SL, Mitchison TJ. 1999. Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. Science 286:971–974.

Mitchison TJ. 1994. Towards a pharmacological genetics. Chem Biol 1:3–6.

Ohlmeyer MHJ, Swanson RN, Dillard LW, Reader JC, Asouline G, Kobayashi R, Wigler M, Still WC. 1993. Complex synthetic chemical libraries indexed with molecular tags. Proc Natl Acad Sci U S A 90:10922–10926.

Oldenburg KR, Zhang JH, Chen T, Maffia A, Blom KF, Combs AP, Chung TDY. 1998. Assay miniaturization for ultra-high throughput screening of combinatorial and discrete compound libraries: A 9600-well (0.2 microliter) assay system. J Biomol Screen 3:55–62.

Pelish HE, Westwood NJ, Feng Y, Kirchhausen T, Shair MD. 2001. Use of biomimetic diversity-oriented synthesis to discover galanthamine-like molecules with biological properties beyond those of the natural product. J Am Chem Soc 123:6740–6741.

Peterson RT, Link BA, Dowling JE, Schreiber SL. 2000. Small molecule developmental screens reveal the logic and timing of vertebrate development. Proc Natl Acad Sci U S A 97:12965–12969.

Rosania GR, Chang YT, Perez O, Sutherlin D, Dong H, Lockhart DJ, Schultz PG. 2000. Myoseverin, a microtubule-binding molecule with novel cellular effects. Nat Biotech 18:304–308.

Schreiber SL. 2000. Target-oriented and diversity-oriented organic synthesis in drug discovery. Science 287:1964–1969.

Tallarico JA, Depew KM, Pelish HE, Westwood NJ, Lindsley CW, Shair MD, Schreiber SL, Foley MA. 2001. An alkylsilyl-tethered, high-capacity solid support amenable to diversity-oriented synthesis for one-bead, one-stock solution chemical genetics. J Comb Chem 3:312–318.

Tan DS, Foley MA, Shair MD, Schreiber SL. 1998. Stereoselective synthesis of over two million compounds having structural features both reminiscent of natural products and compatible with miniaturized cell-based assays. J Am Chem Soc 120:8565–8566.

You AJ, Jackman RJ, Whitesides GM, Schreiber SL. 1997. A miniaturized arrayed assay format for detecting small molecule-protein interactions in cells. Chem Biol 4:969–975.