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High-Throughput Sorting and Placement of One-Bead-One-Compound (OBOC) Libraries from Bulk to Single Wells in Organic Solvent

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Abstract

One-bead-one-compound (OBOC) solid-phase combinatorial chemistry has been used extensively in drug discovery. However, a major bottleneck has been the sorting of individual beads, while still swollen in organic solvent, into individual wells of a microwell plate. To solve this problem we have constructed an automated bead sorting system with integrated quality control that is capable of sorting and placing large numbers of beads in bulk to single wells of a 384-well plate, all in organic solvent. The bead sorter employs a unique, reciprocating fluidic design capable of depositing 1 bead per 1.5 s with an average accuracy of 97%. We quantified the performance of this instrument by sorting over 8,500 beads followed by cleaving the conjugated compound and confirming the chemical identity of each by LC/MS. This instrument should enable more efficient screening of combinatorial small molecule libraries without the need to dry beads or otherwise change chemical environment.

Keywords

Bead sorting; OBOC; organic solvents; high-throughput screening; combinatorial libraries

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Supporting Information Available: Further experimental details as well as supplementary figures for the bead sorting system. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR CONTRIBUTIONS

MWB, CJC, KB, JHL, IF, ABC, and RO performed the experiments. MWB, RO, IF, JVF, and HSC reviewed, analyzed, and interpreted the data. MWB, HSC, and JVF wrote the paper. All authors discussed the results and commented on the manuscript.

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INTRODUCTION

Since the introduction of solid phase supports for the synthetic chemistry of a tetrapeptide by Merrifield in 1963,¹ inert beads have been used to generate hundreds of thousands of unique compounds. Solid supports are commonly used in the 'one-bead-one-compound' (OBOC) approach, and have been used to generate diversity-oriented libraries for nerve targeting dyes,² cancer-targeting peptides,³ and chemical genetics^{4, 5} to name just a few.

Split-pool methods, in combination with equipment designed for high-throughput screenings (HTS), have been a popular approach to drug discovery over the past twenty years.⁶⁻⁸ However, this approach also comes with the unique challenge of segmenting and analyzing each individual solid-phase bead. Typically, a completed library of beads are segmented into individual wells of a 96-, 384-, or 1496-well plate followed by either cleavage and chemical screening, or direct on-bead screening.⁹ The post-synthesis segmentation of a completed library, although easy for the human hand, is time consuming and extremely monotonous. Previously described methods for arraying micron to millimeter sized supports include using a vacuum manifold with dry beads,⁵ droplet deflection using electrostatics,¹⁰ and fluidic deflection of water compatible beads.¹¹ Although each of these methods have their strengths, no system described to date automates the process of sorting beads into multiple microtiter plates while maintaining the bead in organic solvent and providing deposit error checking.

These three issues, automation, solvent compatibility, and error checking, are of practical importance. Bead segmentation processes that rely too heavily on human intervention have bottlenecked the benefit of the diversity-oriented split pool method. The ability to automate sorting over multiple plates removes the limitation of the human hand and the need for human supervision. A bead sorter with chemically compatible fluidics provides flexibility in choosing common, functionalized polystyrene resins that are otherwise difficult to handle in aqueous environments.¹² Lastly, error checking of sorted plates permits verification of the process while simultaneously propagating individual well information that is required for post-sorting analysis and screening.

In this study, we hypothesized that an instrument that incorporated chemically resistant fluidics and quality control algorithms would be capable of sorting OBOC chemical libraries with high speed and high accuracy, without the need to remove the beads from their native chemical environment.

RESULTS AND DISCUSSION

Hardware Integration

As shown in Figure 1 and Supplementary Figures S1 and S2, the bead sorting station was assembled using three pieces of commercially available equipment and a custom bead sorter. The equipment can be categorized by function: management of SBS plates, management of bead sorting, and quality control. A microplate handler (Caliper Twister II) was used for plate management, with its two separate reserve towers used for storage of new plates and storage of processed plates. A TechElan (Mountainside, NJ, www.techelan.com) Bead

Sorter (TeBS), a custom piece of equipment designed for depositing single beads from a suspension of thousands, was selected for bead management. Bead counting was initially accomplished with individual well imaging via a dark-field camera assembled from Microvideo Instruments (Avon, MA). However, the use of a flatbed scanner (Cannon CanoScan 8800F) was chosen to provide whole-plate analysis, which benefits the system with reduced processing time. A translation stage was assembled using two lead screw-driven actuators (Velmex BiSlide) for transporting plates between the areas of plate management, bead management, and quality control. As detailed in Figure 1, the assembly of these four pieces of equipment was the first step in a multistep process, starting from library synthesis and ending with a completed screening assay.

Bead Sorter

The TeBS was selected for bead sorting due to its infinite-loop design. As shown in Figure 2, the beads flow from the source vessel to the receiver vessel while passing through an optically monitored flow cell. A total of ten timing parameters were determined empirically for high accuracy of single bead deposits. These parameters include signal processing triggers which relate to 1) the size of the bead, 2) the separation between beads, and 3) the presence of double beads and bubbles. The most important of these parameters are 1) the bead width time (T_b), which is used to exclude double beads and air bubbles, and 2) the bead follower time (T_f), which negates the deposit if another bead is observed to be following too close to the bead of interest. The custom flow cell of the TeBS is suited for beads between 400–800 μm in size.

Software Integration

The equipment described above was assembled and fully controlled via a custom-made software application written in C# and based on proprietary DLL libraries from National Instruments (NI-DAQ, NI-IMAQdx, and NI-VISION) and Caliper Instruments (iLink Pro). The TeBS communication with the software is accomplished with three digital I/O signals, which reflect the “request bead,” “successful deposit,” and “wait for request” TTL signals to the PLC controller within the TeBS. The “request bead” signal is used by the software to initiate the process of a single bead deposit by the TeBS only if the “wait for request” signal is not active. This “wait for request” TTL is used as feedback to the software to indicate that the TeBS is currently unavailable to deposit a bead, which occurs during routine processes such as vessel switching or fluidic line cleaning. The “successful deposit” signal is the output of the TeBS to confirm a bead deposit, which then triggers the advancement of the well plate by the Velmex stage. Since this station was designed to operate without human intervention, the most challenging aspect was maintaining alignment of the equipment. Positional calibration of the microplate handler was completed through the provided iLinkPro software and bead sorting positional alignment was completed using a 3-well calibration within the station’s software.

Automated Bead Sorting in Organic Solvent

Using the customized bead sorter (Figure 2), nearly 8,500 fluorescein-conjugated beads in DMSO were sorted automatically into 22 384-well plates (Figure 3). The average bead deposit time was between 1.3 and 1.5 s per bead, which is due largely to the ≈ 1.2 s dead

time attributed to the translation of the plate from the previous well. Optical monitoring and bead deflection from the TeBS occurs in a fraction of a second, and is the smallest contribution to total deposit time. An extended bead deposit time can be attributed to any combination of vessel reversal, automated clog recovery, row switching, and plate switching.

Grouped together in the scatter plot of Figure 3 are beads that were deposited in wells following X-Y translation of the plate from one row to the next (i.e., every 25th bead). This is due to the extended travel path between rows and a backlash correction needed for the X-Y stage. Also observed in Figure 3 is the group of beads following a vessel switch or automated clog recovery of the bead sorter, both of which needed approximately twenty seconds for the process to complete. Lastly, the first bead of each plate records a deposit time of ≈ 180 seconds, which is the time the station needs to swap plates using the Twister II plate handler.

Typically, empty wells are the fault of fluidic clogging, in which the automated recovery process will also advance the plate to the next well. These empty wells, marked in yellow in Figure 3, generally appear with a bead deposit time over 5 seconds. A challenge in maintaining high single bead deposits is the alignment of the SBS plate with the TeBS. While arraying beads into a 384-well plate, a minute shift in the translation stage, plate handler, or bead sorter can result in multiple missed wells or absent plates.

In addition, multiple bead deposits in a single well fall within the average deposit times, suggesting that multiple bead deposits were due to beads that circumvented deposit-timing parameters. In order to reduce the number of multiple beads deposited into a single well, the set of timing parameters had to first be determined. The TeBS optical monitor also had to be tuned for a particular bead size in a particular solvent. Of special note, the degree of swelling is dependent on solvent and the properties of the resin. Generally, we found that DMSO will solvate beads and produce an adequate suspension with minimal changes in diameter when compared to other organic solvents such as tetrahydrofuran (THF), dichloromethane (DCM), and dimethyleformamide (DMF). A 10–20% increase in diameter of beads was observed when swollen in dimethyl sulfoxide (DMSO).

Image-Based Bead Quantification

A detailed description of blob analysis and the software workflow can be found in Supplemental Information, especially Figure S3. A typical histogram of bead deposit statistics is shown in Figure 4, and highlights the drop in bead density with later plates. As the bead density in the TeBS vessel decreases, the TeBS requires seconds or several seconds to perform a successful single bead deposit. Overall, the station had a median plate depositing time of 11 minutes and a median single bead deposit accuracy of 97%. Error checking by image processing also carried a unique set of challenges. Round bottom plates resulted in beads settling near the center of the well, which made quantification easier. However, round bottom transparent plates with organic chemical compatibility are not commercially available. The use of a semi-translucent polypropylene plate decreased the reliability of bead counting by dark-field imaging, which resulted in switching to whole plate scanning using a backlight and greatly increased signal to background. The use of flat-

bottom plates encouraged beads to stick to the sides of the well, which decreased reliability of bead quantification.

A representative result from a 22-plate experiment is shown in Figure 5 and includes a single plate deposit and counting chart (A), along with the chemical identification data (D, E). The bead sorting system was able to identify eight empty wells within the 384-well plate (not shown is the first well of the plate as the deposit time was 180 seconds). The post-sorting analyses of the plate by absorbance (UPLC), fluorescence (Gemini XS), and MS (MALDI-TOF and UPLC-TOF) validated the bead sorting counting algorithm as the counting data (A, B), visual identification (C), and analyses (D, E) agree on empty wells. Example chromatograms and mass spectra from UPLC-MS and MALDI-TOF analysis can be seen in the Supplemental Information Figure S5. The presence of multiple beads per well was validated by eye. The above chemical identification process exemplifies the compatibility of SBS plates across multiple automated instruments that are capable of analyses that examine molecular absorbance, fluorescence, light scattering, and exact mass.^{13, 14}

Conclusions

This bead sorting station was developed to automate the process of depositing thousands of individual beads into each well of a 384-well plate while keeping beads in organic solvent. With a median deposit accuracy of 97% and plate processing time of 11 minutes, the bead sorting station can process over twenty plates or approximately 8,500 beads in under five hours. When incorporated into a high-throughput small molecule synthesis and screening program,^{14–16} the instrument we describe has the potential to accelerate drug development and high-throughput screening.

EXPERIMENTAL PROCEDURES

System Qualification

To determine if drug-conjugated beads can be sorted automatically and accurately, we first qualified the station using fluorescein conjugated to 400 – 450 μm CIT polystyrene resin. Fluorescein was chosen for conjugation as it is a small molecule that can be easily identified post-sorting by absorbance, fluorescence, and mass spectroscopy. Approximately 8,500 fluorescein-conjugated beads were prepared in DMSO using an acid cleavable linker.

Fluorophore Conjugation and Cleavage

Fluorescent PS beads were prepared by first dissolving 0.798 g of fluorescein in 30 mL of 2:1 DCM:DMF then adding 1.11 mL of DIEA. A total of 1 g of PS-CIT (0.65 mmol/g) beads were added and left to react overnight at room temperature. The reaction scheme is shown in Figure S4. After 16 h, the mixture was filtered and washed with methanol, DMF, then DCM (3 $\times$ 5 mL each) to yield the final product. Post-sorting cleavage of the fluorophore was accomplished using with the addition of 1M HCl solution directly into each well containing organic solvent. Additional information on the fluorophore cleavage can be found in the Supplemental Information.

Statistical Data

Statistics on the operation of the bead sorting station was accomplished through a deposit log created by the control software. Each well was labeled with a numerical identification number (WELL_ID) and associated statistics for each WELL_ID are the deposit time and quality control (QC) counting data. The deposit time is the amount of time between “successful deposit” signals of the TeBS. The well deposit time takes into account the dead time of the X-Y translation from the previous well, the optical monitoring time, and other dead time such as automated clog detection/recovery, or vessel switching.

Image-Based Bead Quantification

Post-sorting imaging of the well plates is used for QC, which quantifies the number of beads in each well. Images were collected either on a per well basis using a dark-field camera (Microvideo Instruments) or a per plate basis using a Canon flatbed scanner equipped with a back light. The image-processing algorithm utilizes feature detection and a counting classification, which includes measuring any color attributes of the bead (Color Plane Extraction) and extracting a ROI for each well. The image is then processed using a binary threshold for segmentation, analyzed, and discriminated based on the “centroid,” “area,” and “circularity” features of each blob particle. Finally, classification of the blobs is accomplished through an empirically determined area and circularity relating to the single, double, or triple beads per well. Counting results are then merged with deposit timing results acquired from the TeBS. Example images and a counting flow-chart are contained in the Supplemental Information Figure S3.

Chemical Identification

After sorting into individual wells, the chemical identity of each bead was determined by preparing a parent plate consisting of conjugated dye cleaved from the resin using an automated liquid handler (Caliper SciClone ALH2000). Child plates were generated by an automated process using the same liquid handler by diluting and transferring the parent plate to desired concentrations suitable for UPLC-MS (Waters Acquity UPLC – Xevo G2 QTOF), MALDI-TOF (Bruker MALDI-TOF-TOF), and plate scanning (Gemini XS, Molecular Devices, Sunnyvale, CA). In the case of the child plate prepared for UV-VIS absorbance and fluorescence, sodium hydroxide was used to increase the pH to a suitable, basic environment. The fluorophore was identified during LC-MS by fluorescence at 521 nm and ESI- at 331 m/z. Plate scanning spectra were collected between 480 – 680 nm with an excitation of 450 nm. MALDI-TOF identification was completed with observed negative ions at 287 m/z and 353 m/z, corresponding to $[M-COOH]^-$ and $[M-2H+Na]^-$. Additional analysis details are contained in Supplemental Information Figure S5.

Chemicals, Reagents, and Disposables

Standard SBS sized 384-well plates with 100 μ L round bottom polypropylene wells were used in this study (Corning, CoStar 3657). Chloro-(2'-chloro)trityl (CIT) polystyrene resin was purchased through Rapp Polymere (Tuebingen, Germany) in sizes of 400 – 450 μ m (catalog #H40045033) and 500 – 560 μ m (catalog #H50056033) before swelling in organic solvent. Fluorescein was purchased from Sigma-Aldrich (catalog #32615-25G-R).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Design and functionality of the automated bead sorting system: Single-bead/single-well sorting was performed after solid phase chemical library synthesis on 400–500 μm diameter beads. Post-cleavage analysis of processed plates may include chemical identification or cell study assays after compound cleavage from the beads. The bead sorting system is comprised of a microplate handler, a camera for quality control, X-Y stage, and a bead sorter arranged as shown.

Figure 2.

Bead fluidics and optical sensing: Beads pass through an optically-monitored flow cell while being transported in organic solvent from the source vessel (#1) to the receiver vessel (#2). The optical sensor data is used in conjunction with single bead deposit parameters (T_b , T_f , etc.) to deflect individual beads from the suspension into a microtiter plate well below. Shown are examples of A) single bead deposit response, B) a pair of beads travelling too close for a successful deposit, and C) an air bubble in the fluidic line. At the point when the source vessel no longer contains fluid, the vacuum/pressure levels reverse and the receiver vessel becomes the source vessel (and vice versa). This process is repeated until the predetermined number of plates is complete or no successful single bead deposits can be accomplished.

Figure 3.

Sorting and deposit statistics: Shown are the bead sorting and deposit statistics for $\approx 8,500$ 400–450 μm CIT beads conjugated to fluorescein in DMSO deposited into 22 384-well plates. Bead deposit time is measured as the time between two “deposit successful” signals from the bead sorter and can include 1) dead time from the X-Y stage advancing to the next well, 2) monitoring time until a single bead condition is met, 3) dead time from the X-Y stage advancing to the next row, 4) vessel reversal time, 5) clog recovery time, and 6) plate switching time. Expressed through the color of each deposit spot is the image-processed counting result for that well, which may be empty, contain a single bead, or contain multiple beads. In addition, every 25th well is highlighted in purple outline to indicate the extra time needed to perform a plate row switch.

Bead deposit time histogram: Shown is the bead deposit time histogram for the 22 384-well plates from Figure 3. Note a continuous deposit time shift from shorter (0–2 s) to longer deposit times (2–5 s) due to a decrease in bead density. Batch plate counting and plate time results show a median plate time of 11 min and average single bead deposit accuracy of $96.7 \pm 1.9\%$.

Figure 5. Propagation of bead sorting data to post-cleavage analysis. A) Bead deposit time chart for a single plate and its B) corresponding plate image used in the quantification of beads per well. The C) post cleavage plate is shown to provide a visual reference for confirmation of D) UPLC-TOF & plate reader analysis, and E) MALDI-TOF analysis.