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

Mark W. Bordo,[†] Rafiou Oketokoun,[‡,§] Conor J. Cross,[‡,§] Kai Bao,[‡,§] Jeong Heon Lee,[‡,§] Ilya Feygin,[⊥] Alex B. Chang,[⊥] John V. Frangioni,[†,§,▽] and Hak Soo Choi*[,‡,§]

[†]Curadel, LLC, 377 Plantation Street, Worcester, Massachusetts 01605, United States

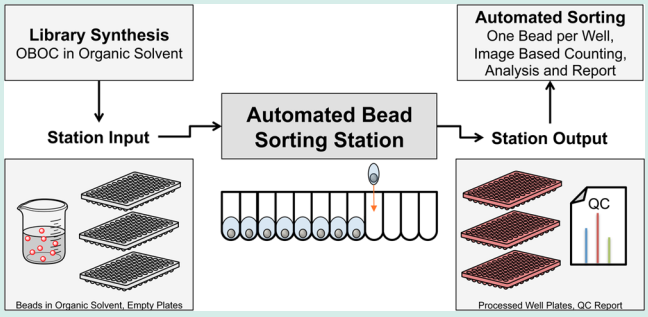
[‡]Robotic Chemistry Group, Center for Molecular Imaging, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215, United States

[§]Division of Hematology/Oncology, Department of Medicine and [▽]Department of Radiology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215, United States

[⊥]TechElan LLC, 853 Hillside Ave., Mountainside, New Jersey 07092, United States

Supporting Information

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 an organic solvent. The bead sorter employs a unique, reciprocating fluidic design capable of depositing 1 bead every 1.5 s, with an average accuracy of 97%. We quantified the performance of this instrument by sorting over 8500 beads, followed by cleaving the conjugated compound and confirming the chemical identity of each by liquid chromatography/mass spectrometry (LC/MS). This instrument should enable more efficient screening of combinatorial small molecule libraries without the need to dry beads or otherwise change the chemical environment.



KEYWORDS: *bead sorting, OBOC, organic solvents, high-throughput screening, combinatorial libraries*

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 they 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 20 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 micrometer- 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 an organic solvent and providing deposit error checking.

These three issues are of practical importance: automation, solvent compatibility, and error checking. Bead segmentation processes that rely too heavily on human intervention have restricted the benefits 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

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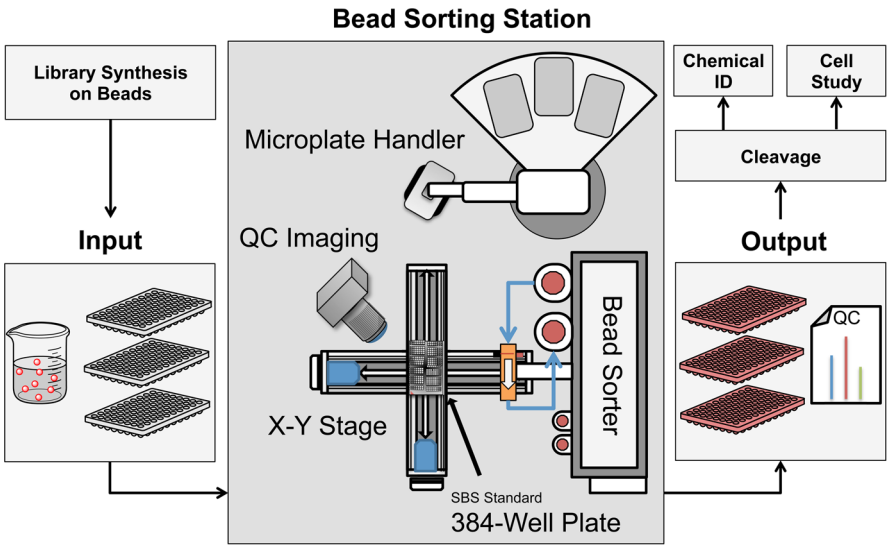

Figure 1. Design and functionality of the automated bead sorting system. Single-bead/single-well sorting was performed after solid phase chemical library synthesis on beads 400−500 μm in diameter. Post-cleavage analysis of processed plates may include chemical identification or cell study assays after compound cleavage from the beads. The bead sorting system is comprised of a microplate handler, a camera for quality control, an X−Y stage, and a bead sorter, arranged as shown.

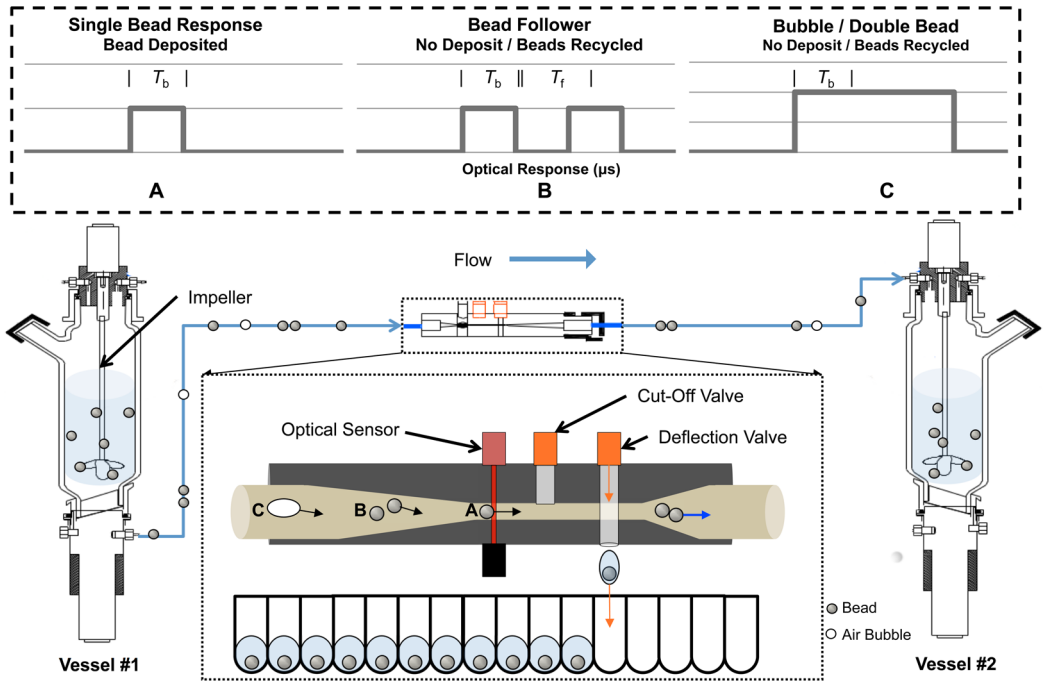

Figure 2. Bead fluidics and optical sensing. Beads pass through an optically monitored flow cell while being transported in an organic solvent from the source vessel (Vessel #1) to the receiver vessel (Vessel #2). The optical sensor data are used in conjunction with single bead deposit parameters (T_b, T_f, etc.) to deflect individual beads from the suspension into a microtiter plate well below. Shown are examples of (A) a single bead deposit response, (B) a pair of beads traveling 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.

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 (QC) 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.

Hardware Integration. As shown in Figure 1 (and in Figures S1 and S2 in the Supporting Information), 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; its two separate reserve towers were used for the storage of new plates and processed plates. A TechElan Bead Sorter (TeBS) (TechElan, Mountainside, NJ, www.techelan.com), which is a custom piece of equipment designed for

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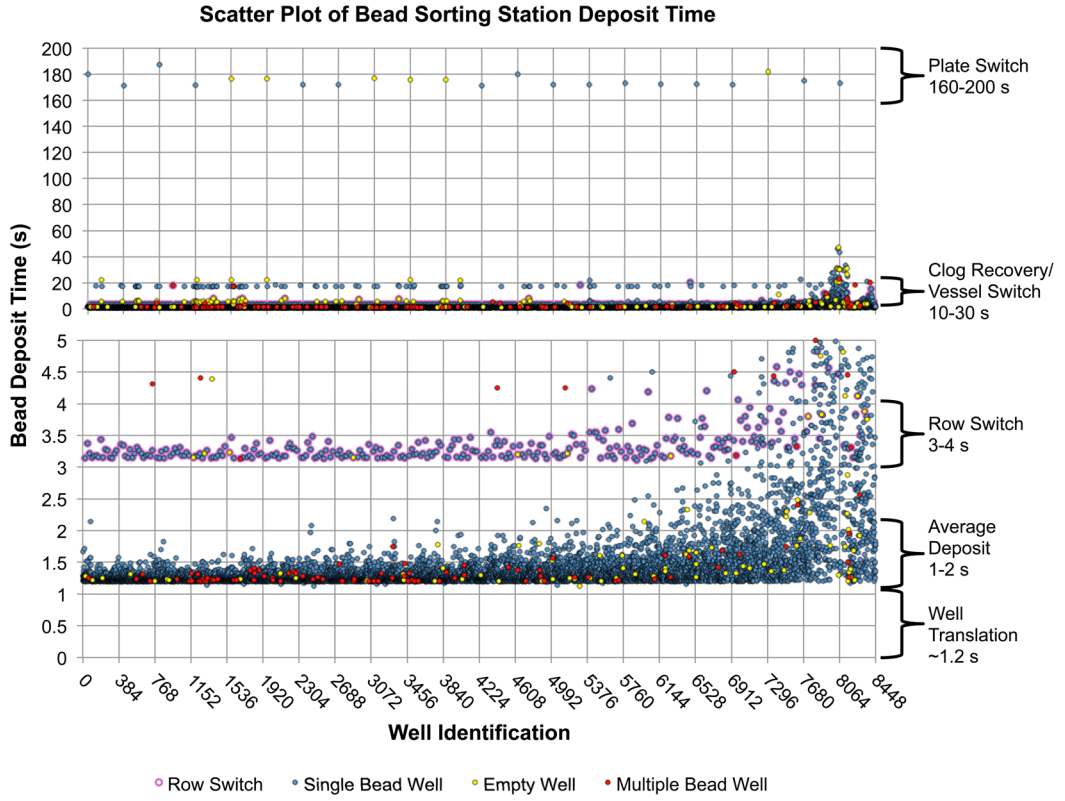

Figure 3. Sorting and deposit statistics. Shown are the bead sorting and deposit statistics for ~8500 400−450 μm ClT beads conjugated to fluorescein in DMSO deposited into 22 384-well plates. Bead deposit time is measured as the time between two "deposit successful" signals from the bead sorter and can 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.

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 (Canon, Model 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 because of 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 10 timing parameters were determined empirically for high accuracy of single bead deposits. These parameters include signal processing triggers that are related 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 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 three-well calibration within the station's software.

Automated Bead Sorting in Organic Solvent. Using the customized bead sorter (Figure 2), ~8500 fluorescein-conjugated beads in DMSO were sorted automatically into 22 384-well plates (Figure 3). The average bead deposit time was 1.3−1.5 s per bead, which is due largely to the ~1.2 s dead

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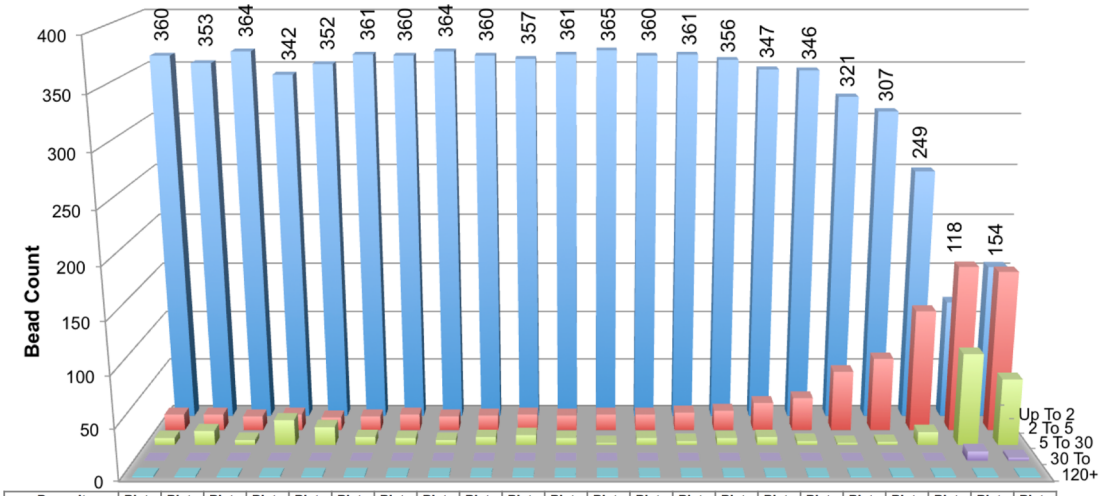

Deposit Range (s)	Plate 01	Plate 02	Plate 03	Plate 04	Plate 05	Plate 06	Plate 07	Plate 08	Plate 09	Plate 10	Plate 11	Plate 12	Plate 13	Plate 14	Plate 15	Plate 16	Plate 17	Plate 18	Plate 19	Plate 20	Plate 21	Plate 22
0–2	360	353	364	342	352	361	360	364	360	357	361	365	360	361	356	347	346	321	307	249	118	154
2–5	16	16	14	16	13	14	16	14	15	16	15	16	16	18	20	28	33	60	73	121	165	160
5–30	7	14	5	25	18	8	7	5	8	10	7	2	7	4	7	8	4	2	3	13	91	66
30–120	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	3
120+	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
# Single Beads	378	750	1127	1479	1843	2213	2590	2961	3332	3702	4077	4457	4829	5203	5578	5955	6331	6708	7085	7456	7819	8173
% Single Beads Sorted	4.5	8.9	13.3	17.5	21.8	26.2	30.7	35.0	39.4	43.8	48.3	52.8	57.2	61.6	66.0	70.5	74.9	79.4	83.9	88.3	92.6	96.7

																							Mean	Median	STDEV
% Empty	1.3	2.1	1.0	4.4	2.9	1.8	0.8	1.3	1.6	2.1	1.3	0.5	1.8	1.8	1.6	1.3	1.3	1.3	1.0	2.3	4.9	5.5	2.0	1.6	1.3
% Single	98.4	96.9	98.2	91.7	94.8	96.4	98.2	96.6	96.6	96.4	97.7	99.0	96.9	97.4	97.7	98.2	97.9	98.2	98.2	96.6	94.5	92.2	96.7	97.1	1.9
% Multiple	0.3	1.0	0.8	3.9	2.3	1.8	1.0	1.8	1.8	1.6	1.0	0.3	1.3	0.8	0.8	0.5	0.8	0.3	0.8	1.0	0.5	2.3	1.2	1.0	0.9
Time (min)	10.4	11.4	9.8	13.5	12.5	10.8	10.2	9.9	10.6	11.3	10.7	10.2	10.6	10.4	11.1	11.4	10.9	11.3	12.1	14.7	34.3	25.6	12.9	11.0	5.8

Figure 4. 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 an average single bead deposit accuracy of 96.7% ± 1.9%.

time, which is attributed to the translation of the plate from the previous well. Optical monitoring and bead deflection from the TeBS occurs within a fraction of a second, and this is the smallest contribution to the 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 required ∼20 s for the process to complete. Lastly, the first bead of each plate records a deposit time of ∼180 s, 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 s. One 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 first had to 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 dimethylformamide (DMF). A 10%–20% increase in bead diameter 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 the Supporting Information, especially Figure S3. A typical histogram of bead deposit statistics is shown in Figure 4, which highlights the decrease in bead density as the plate number increases. 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 deposition time of 11 min 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 semitranslucent polypropylene plate decreased the reliability of bead counting by dark-field imaging, which resulted in a switch to whole-plate scanning using a backlight and greatly increased the signal, relative to the background. The use of flat-bottom plates

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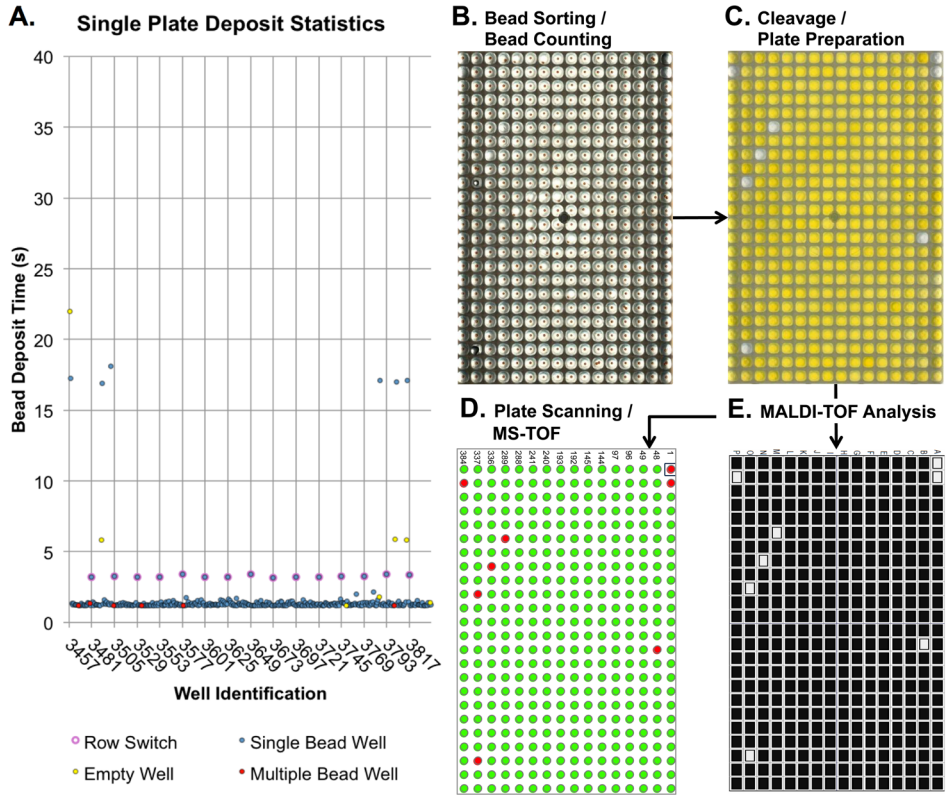

Figure 5. Propagation of bead sorting data to post-cleavage analysis: (A) bead deposit time chart for a single plate and (B) its corresponding plate image, which was used in the quantification of beads per well. The post-cleavage plate (shown in panel C) provides a visual reference for confirmation of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

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

A representative result from a 22-plate experiment is shown in Figure 5; it includes a single plate deposit and counting chart (Figure 5A), along with the chemical identification data (Figures 5D and 5E). The bead sorting system was able to identify eight empty wells within the 384-well plate (the first well of the plate is not shown, because the deposit time was 180 s). The post-sorting analyses of the plate by absorbance (ultra performance liquid chromagraphy (UPLC)), fluorescence (Gemini XS), and mass spectroscopy (MS) (matrix-assisted laser desorption/ionization−time of flight (MALDI-TOF) and UPLC-TOF analyses) 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 Figure S5 in the Supporting Information. 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]

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 min, the bead sorting station can process over 20 plates (or ∼8500 beads) within less than 5 h. When incorporated into a high-throughput small-molecule synthesis and screening program,[14−16] the instrument that 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 ClT polystyrene resin. Fluorescein was chosen for conjugation, because it is a small molecule that can be easily identified post-sorting by absorbance, fluorescence, and mass spectroscopy. Approximately 8500 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 and then adding 1.11 mL of DIEA. A total of 1 g of PS-ClT (0.65 mmol/g) beads were added and allowed to react overnight at room temperature. The reaction scheme is shown in Figure S4 in the Supporting Information. After 16 h, the mixture was filtered and washed with methanol, DMF, then DCM (3 × 5 mL each) to yield the final product. Post-sorting cleavage of the fluorophore was accomplished with the addition of 1 M HCl solution directly into each well containing organic solvent. Additional information on the fluorophore cleavage can be found in the Supporting Information.

Statistical Data. Statistics on the operation of the bead sorting station were obtained through a deposit log created by the control software. Each well was labeled with a numerical identification number (WELL_ID), and the associated statistics for each WELL_ID are the deposit time and quality control (QC) counting data. The deposit time is the amount of time

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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 an 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 provided in Figure S3 in the Supporting Information.

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 hander (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-MS at 331 m/z. Plate scanning spectra were collected at wavelengths of 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 provided in Figure S5 in the Supporting Information.

Chemicals, Reagents, and Disposables. Standard SBS-sized 384-well plates with 100 μL round-bottom polypropylene wells were used in this study (Corning, CoStar 3657). Chloro-(2′-chloro)trityl (ClT) polystyrene resin was purchased through Rapp Polymere (Tuebingen, Germany) in sizes of 400−450 μm (Catalog No. H40045033) and 500−560 μm (Catalog No. H50056033) before swelling in an organic solvent. Fluorescein was purchased from Sigma−Aldrich (Catalog No. 32615-25G-R).

■ ASSOCIATED CONTENT

Supporting Information

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 INFORMATION

Corresponding Author

*Address: 330 Brookline Avenue, Room SL-436A, Boston, MA 02215. Tel.: 617-667-6024. Fax: 617-667-0214. E-mail: hchoi@bidmc.harvard.edu.

Author Contributions

M.W.B., C.J.C., K.B., J.H.L., I.F., A.B.C., and R.O. performed the experiments. M.W.B., R.O., I.F., J.V.F., and H.S.C. reviewed, analyzed, and interpreted the data. M.W.B., H.S.C., and J.V.F. wrote the paper. All authors discussed the results and commented on the manuscript.

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Notes

The authors declare the following competing financial interest(s): TeBS Bead Sorter is commercially available from TechElan, a for-profit company. Ilya Feygin is the Vice President of Engineering at TechElan.

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