Bigfoot Spectral Cell Sorter

High-throughput plate sorting

Introduction

High-speed electrostatic droplet sorting is the standard technique for cell purification across a wide breadth of research areas in the biological sciences and beyond. Purified cells are utilized for an array of downstream experiments, including functional assays, DNA analysis, gene expression analysis, and cell line development. Some of these key applications, especially gene expression analysis through RNA sequencing and clonal cell line development, require direct deposition of single cells into multiwell plates. Microtiter plate sorting is a powerful capability that takes advantage of the inherent single-cell resolution of flow cytometry and droplet-based cell sorting, but it presents unique challenges. Specifically, cells must be accurately and precisely targeted into each well to ensure that cells are deposited directly into potentially small volumes of capture fluid.

Plate sorting is accomplished through movement of a stage that holds the plate, positioning it directly under a sort stream that will deliver a cell into each well. Because of the requirement for precise targeting, the plate deposition mechanism requires careful calibration and alignment. Alignment is commonly verified by sorting a small number of droplets onto the cover of the plate and ensuring that the drops are positioned in the center of the well as viewed from the top of the plate. However, this method is imperfect, especially for deposition into PCR plates. These plates have conical wells, so the actual target at the bottom of the well is much smaller than at the top of the plate. Moreover, the top of the plate can be as much as 20 mm above the bottom. The side stream trajectory on most cell sorting instruments is angular and not perpendicular to the horizontal plane of the plate; consequently, targeting the center of the well at the top of the plate may not ultimately result in cell deposition in the buffer at the bottom of the well.

Therefore, alternative methods have been developed to verify plate alignment. One such method, developed by Rodrigues and Monard [1], utilizes horseradish peroxidase (HRP) to catalyze a colorimetric reaction, which indicates whether a droplet has been deposited into a well. In this method, a plate is filled with a small volume of buffer containing the colorless substrate 3,3′,5,5′-tetramethylbenzidine (TMB), and beads suspended in a solution containing HRP are sorted into each well. TMB turns blue after reacting with HRP, so any color change after sorting indicates successful droplet deposition.

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The Invitrogen™ Bigfoot Spectral Cell Sorter is a cutting-edge, high-parameter instrument that features a multitude of cell-sorting advancements. Innovations in plate deposition ensure unprecedented accuracy, recovery, and speed down to single-cell sorting. These improvements include built-in stream calibration and drop delay, built-in media detection imaging that permits accurate plate setup and verification, highly robust hardware for precise single-droplet targeting with minimal adjustment, and straight-down sorting for maximum deposition accuracy. In addition, the sort output hardware facilitates maximum flexibility, permitting deposition into plates with up to 1,536 wells and even nonstandard devices like 10x™ chips [2]. Finally, and most impressively, the Bigfoot Spectral Cell Sorter is capable of four-way sorting into 96-well plates and eight-way sorting into 384-well plates for unprecedented speed, far surpassing currently available hardware. This multiway plate sorting feature is unavailable on any other cell sorter.

A variety of tests using the HRP method described above were performed on the Bigfoot Spectral Cell Sorter to test deposition accuracy and precision, robustness, and speed of the hardware. These tests demonstrate the Bigfoot Spectral Cell Sorter's revolutionary capabilities and utility in any setting where plate sorting is required.

Materials

Lyophilized, salt-free HRP (Gold Biotechnology, MO) was reconstituted in Hanks' Balanced Salt Solution (HBSS) and diluted to a working solution of 5 mg/mL. A bead and HRP mixture was prepared by adding 2 µL of 4.5 micron Polybead™ Carboxylate Microsphere suspension (4.99 x 10^8 particles/mL, Polysciences Inc, PA) to 0.5 mL of HRP working solution.

Multiwell plates were prepared as follows: 50 µL of TMB High Sensitivity Substrate Solution (BioLegend, CA) were added to each well of a 96-well Thermo Scientific™ Armadillo™ PCR amplification plate (Thermo Fisher Scientific, MA), and 10 µL TMB High Sensitivity Substrate Solution (BioLegend, CA) were added to each well of a 384-well Thermo Scientific™ Armadillo™ PCR amplification plate (Thermo Fisher Scientific, MA). Plates were prepared and stored at 4°C in the dark until utilized.

Methods

To ensure optimal sort conditions, the Bigfoot Spectral Cell Sorter was set up and calibrated through automated software processes, which include fluidics preparation, droplet and deflection setup, optical alignment, and drop charge delay calculation. To ensure optimal alignment between the sort streams and the plate, a test pattern was briefly activated to deposit small puddles of droplets onto aluminum plate-sealing foils. The alignment was adjusted accordingly until all puddles were centered across the wells. Subsequently, with the aluminum plate-sealing foils still fixed to the plates, five events were sorted above each well across the plate, and the positions of the resultant puddles were used to further fine-tune the alignment. The same procedure was used for both 96- and 384-well plates.

In order to thoroughly characterize the well deposition performance of the instrument, using a published evaluation method [1], the sort was configured so the number of events sorted per well was varied across the plates. Sort decisions for the 96-well PCR plates were configured so that four target events were sorted into row A, three target events into row B, two target events into row C, one target event into row D, zero target events into row E, and one event into rows F, G, and H. Sort decisions for the 384-well PCR plates were configured so that one target event was sorted into rows A, C, E, G, I, K, M, O; and zero target events into rows B, D, F, H, J, L, N, P.

As previously described, the prepared microsphere suspension containing HRP was acquired on the Bigfoot Spectral Cell Sorter. The primary 488 nm forward scatter (FSC-A) and primary 488 nm side scatter (SSC-A) parameter PMT voltages were adjusted to place the bead population at a median channel of 128 on both axes. A rectangular gate was used to define the bead population based on FSC and SSC signals, and doublets were excluded through gating on an FSC-A x FSC-H plot (Figure 1).

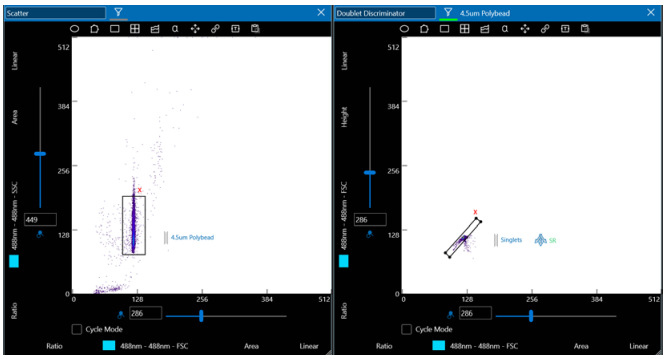

Figure 1. Representative forward scatter (FSC-A) versus side scatter (SSC-A) density plot alongside a representative doublet discrimination density plot (FSC-A x FSC-H). The sort gate was defined by a region in the FSC-A x SSC-A plot to identify the Polybead Carboxylate Microspheres, and a region in the FSC-A x FSC-H plot to define single beads.

All sorts were performed at 150 events/second using single-cell sort mode in which one drop was sorted and both the target droplet and the adjacent quarters of neighboring droplets were required to be free of any other events. Sorts were performed using the unique multistream plate sorting feature of the Bigfoot Spectral Cell Sorter: four side streams were utilized for 96-well plate sorting (Figure 2) and eight side streams for 384-well plate sorting (Figure 3). This strategy allows completion of a plate with only two passes across the plate, achieving unparalleled speed. The elapsed time to complete each plate was recorded after each sort. Plates were incubated at room temperature in the dark for a minimum of 15 minutes prior to being photographed to allow the HRP reaction to progress and the resultant color to develop. A total of five plates of each plate type were sorted with no realignment between plates. All plates produced similar results.

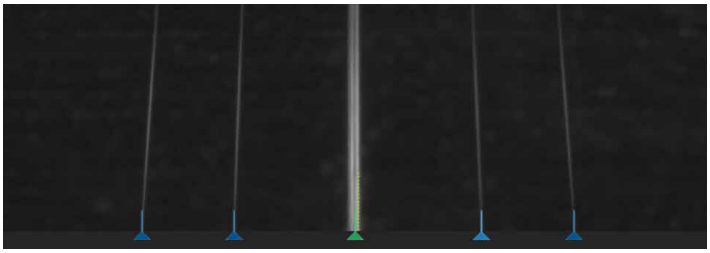

Figure 2. Test pattern with alignment targets of four-way plate sorting mode for 96-well plates.

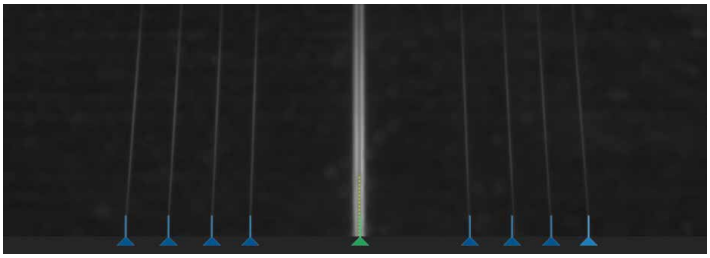

Figure 3. Test pattern with alignment targets of eight-way plate sorting mode for 384-well plates.

Results

The colorimetric reaction of HRP and TMB can be verified visually on all plates when sorting a single droplet containing one event suspended in HRP solution. As expected, the reaction intensifies as the number of sorted droplets increases. Figure 4 shows an example of one of the 96-well PCR plates post-sort and post-incubation. Row A contains four sorted droplets of HRP and exhibits a vivid, dark blue color when reacted with the TMB substrate. The gradient of the colorimetric reaction is increasingly paler in subsequent rows B (three droplets), C (two droplets), and D (one droplet). Row E was assigned zero events in the sort logic which resulted in clear, unreacted TMB after 30 minutes of incubation. Rows F, G, and H were all assigned one target event in the sort logic and contain a similar pale blue color to row D.

The elapsed time to complete each 96-well plate was recorded from the time the sort was executed in the software until the plate was back in its origin position for user retrieval. Table 1 shows the recorded sort times across all five 96-well plates using four-way sorting as described in the methods. The average sort time was less than eight seconds per plate. All five 96-well plates were sorted sequentially with no realignment between plates. The total sort time for all plates, excluding the time required to install the plate on the instrument, initiate the sort, and remove the plate from the instrument, was 39.46 seconds.

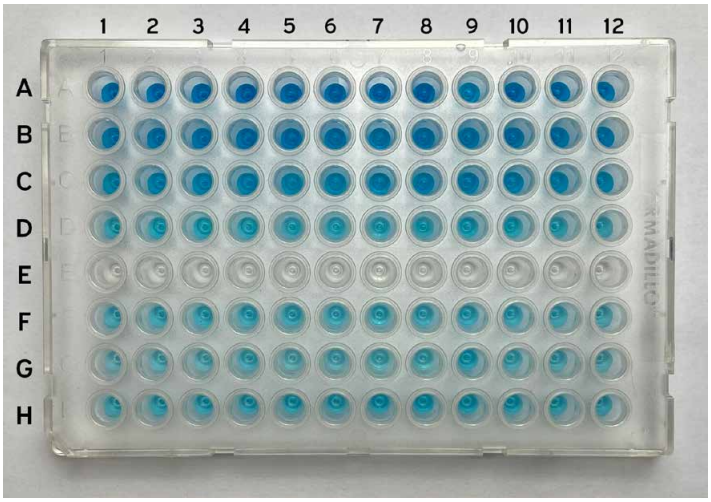

Figure 4. 96-well plate following multidroplet event sorting suspended in HRP solution. TMB substrate turned blue, post-sort, after 30 minutes of incubation in the dark.

Table 1: Elapsed sort time to complete a full 96-well plate using multiple droplet four-way sorting.

96-well plate number	Elapsed sort time
Plate 1	8.17 sec
Plate 2	8.08 sec
Plate 3	7.90 sec
Plate 4	7.47 sec
Plate 5	7.84 sec
Average	7.89 sec

Figure 5 shows an example of one 384-well PCR plate with single droplets sorted across the plate in alternating rows. Rows A, C, E, G, I, K, M, and O all contain one sorted droplet as evident with the dark blue color of the reacted TMB. Rows B, D, F, H, J, L, N, and P, which were all assigned zero events in the sort logic, contain clear, unreacted TMB after 15 minutes of incubation.

Similar to the 96-well plates, the elapsed time to complete each 384-well plate was recorded from the time the sort was executed in the software until the plate was back in its origin position for user retrieval. Table 2 shows the recorded sort times across five plates using eight-way sorting as described in the methods. The average sort time was less than 11 seconds per plate. All five 384-well plates were sorted sequentially with no realignment between plates. The total sort time for all plates, excluding the time required to install the plate on the instrument, initiate the sort, and remove the plate from the instrument, was 52.08 seconds.

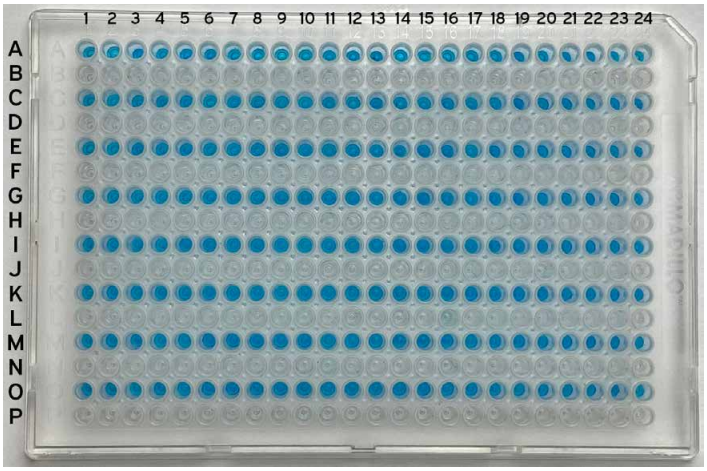

Figure 5. 384-well plate following single droplet event sorting in HRP solution in alternate rows. TMB substrate turned blue, post-sort, after 15 minutes of incubation in the dark.

Table 2: Elapsed sort time for single-droplet eight-way sorting into 384-well plates.

384-well plate number	Elapsed sort time
Plate 1	10.51 sec
Plate 2	10.38 sec
Plate 3	10.20 sec
Plate 4	10.12 sec
Plate 5	10.87 sec
Average	10.42 sec

Discussion and conclusion

The importance of single-cell plate deposition by flow cytometric cell sorting across a variety of research areas is unequivocal. However, this application can present unique challenges. In particular, nanoliter-sized droplets must be deposited with high accuracy and precision into small volumes of capture fluid as low as 2 µL. The predominant factor that impacts the efficiency of this process is the design of the instrumentation. The stability of the fluidics, the robustness of the mechanical arm that holds the plate, and the speed of plate movement all influence the quality of the experimental results of a plate-based sort. One issue common to other plate sorting devices is the unpredictable movement of the sorting mechanism, which results in misalignment of the plate to the sort stream and empty wells on the plate. Another issue is the speed of the plate mechanism. Slow movement extends the sort time and results in the evaporation of capture buffer from the wells. This reduces the capture efficiency, especially when working with small collection volumes.

In contrast, the Bigfoot Spectral Cell Sorter was designed to overcome these challenges. In particular, the mechanism responsible for plate movement was engineered to accurately deposit droplets within 0.175 mm or 10% of the surface area of a well of a 1,536-well plate. Furthermore, the device was designed to travel the entire length and width of a microwell plate in 0.5 seconds, with minimal lag during transition between wells. Both the positional accuracy and high speed of the plate sorter is the product of a custom-designed linear actuator system, fine-pitch lead screws, high-torque motors, and an intelligent spatial sensing system.

Additionally, the deflection system was conceived to achieve the most stable droplet deflection possible. This was accomplished by optimizing both the deflection path and droplet charging. The deflection distance and charge plate configuration were designed to allow for highly accurate deflection over the full space required for precise sorting while maintaining low droplet charge voltages. Increasing the magnitude of charge on droplets can be problematic due to the tendency of droplets to combine by charge attraction, therefore minimizing charge and maximizing the deflection distance to facilitate highly stable and accurate deposition. Furthermore, the hardware architecture was optimized to maximize electronics speed by executing all operations in real-time hardware control. The entire list of operations is programmed ahead of the sort, eliminating the need for the workstation and operating system to watch and respond to status changes. Moreover, from a data processing perspective, the architecture is powerful enough to handle very high event and sort rates with zero electronic aborts.

This demonstrates that the Bigfoot Spectral Cell Sorter offers extremely robust and high-performance plate sorting capabilities, providing consistent deposition efficiency and cell recovery at high speeds for optimal input into downstream experiments. Using the HRP method, with visual confirmation of droplet deposition through the colorimetric conversion of TMB substrate with HRP, it has been shown that a single droplet can be sorted in small volumes into both 96-well PCR plates and 384-well PCR plates with 100% targeting accuracy. Furthermore, it has been demonstrated here that plates can be sorted with unprecedented speed at averages of less than 8 seconds for 96-well plates and less than 11 seconds for 384-well plates. High speed is accomplished through the unique multiway plate sorting capability of the Bigfoot Spectral Cell Sorter, which is available on no other instrument. This feature facilitates the generation of four- and eight-sort streams for 96-well and 384-well plates, respectively, which limits the number of times the plate mechanism must move during the sort, thus reducing the total sort time. In contrast, other available cell sorters can generate only one side stream during plate sorting, and the required plate movement adds time to sort duration.

Moreover, these results demonstrate the robustness of the plate sorting mechanism. Alignment was performed only once before the sort, but the alignment was stable over the course of five sorts even for the difficult targets of 384-well plates. This shows that researchers can be confident that they will achieve the same deposition efficiency for the first and last plates, without regularly rechecking the stream alignment.

In all, these results show that the Bigfoot Spectral Cell Sorter has been thoughtfully and thoroughly designed to provide unparalleled benefits for plate sorting applications. Researchers can sort with confidence knowing that the Bigfoot Spectral Cell Sorter provides consistent sorting through precision, accuracy, robustness, and speed. Furthermore, the usability of the system, through its automation and software, allows operators with all levels of knowledge to use the system with confidence. The Bigfoot Spectral Cell Sorter offers capabilities beyond any other cell sorter, should enhance the workflow of many applications, and will be an asset to research laboratories now and in the future.

References

1. Rodrigues, O.R. and Monard, S. (2016), A rapid method to verify single-cell deposition setup for cell sorters. *Cytometry*, 89: 594–600. doi:10.1002/cyto.a.22865.
2. 10x Genomics, 2020, Products, viewed 4 April 2020, **https://www.10xgenomics.com/products**.

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