Cell sorting technologies

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 applications, especially gene expression analysis using RNA sequencing and clonal cell line development, require direct deposition of single cells into multi-well plates. Microplate 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 by 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.

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 imaging that permits accurate plate setup and verification, highly robust hardware for precise single-droplet targeting with minimal adjustment, and a straight-down sorting option 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 Petri dishes and 10x Genomics™ chips [2]. Finally, and most impressively, the Bigfoot Spectral Cell Sorter is capable of four-way sorting into 96- and 384-well plates for unprecedented speed, far surpassing currently available hardware. This multi-way 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. Chinese hamster ovary (CHO) cells were cultured in RPMI medium with 10% FBS and 1X penicillin–streptomycin until confluent. Cells were detached from the culture flask using Gibco™ TrypLE™ Express Enzyme and neutralized using the cell culture medium. Cells were centrifuged at 400 x *g* for 5 minutes at 4°C and subsequently stained with Invitrogen™ Hoechst™ 33342 dye solution for 20 minutes in the dark following the manufacturer's recommendation. Cells were washed with cold PBS and resuspended in 5 mg/mL HRP-HBSS working solution for cell sorting.

Multi-well plates were prepared as follows: 50 µL of TMB High Sensitivity Substrate Solution (BioLegend, CA) was added to each well of a 96-well Thermo Scientific™ Armadillo™ PCR amplification plate, 10 µL of TMB High Sensitivity Substrate Solution was added to each well of a 384-well Thermo Scientific™ Armadillo™ PCR amplification plate, and 5 µL of TMB High Sensitivity Substrate Solution was added to each well (or specific wells for advanced sorting mode) of a 1536-well Thermo Scientific™ Nunc™ microplate. Plates were prepared and stored at 4°C in the dark until used. 96-well and 384-well Thermo Scientific™ Nunc™ clear cell culture–treated plates were set aside for assessing cell occupancy using the Invitrogen™ EVOS™ M7000 Imaging System. 1536-well Greiner Bio-One™ SensoPlate™ glass-bottom microplates were also set aside for assessing cell occupancy. TMB High Sensitivity Substrate Solution was not added to the cell culture–treated multi-plates or the 1536-well glass-bottom microplates used for the cell occupancy assessment.

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, the output media calibration wizard was used to accurately target the side streams to the well positions. A test pattern was briefly activated to deposit small puddles of droplets onto the output calibration plate targets, and the stream alignment was adjusted until all puddles were centered across the wells. Subsequently, with aluminum plate-sealing foils fixed to the plates, a test sort using sheath droplets was sorted across the plate, and the positions of the resultant puddles were used to further fine-tune the alignment. The same procedure was used for alignment of 96- and 384-well PCR plates, 96- and 384-well cell culture–treated plates, and all 1536-well microplates.

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 target event into rows F, G, and H. The sorts were repeated using 96-well cell culture–treated plates to assess cell occupancy using the EVOS M7000 Imaging System.

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, and O; and zero target events into rows B, D, F, H, J, L, N, and P using the same published evaluation method [1]. The sorts were repeated using 384-well cell culture–treated plates to assess cell occupancy using the EVOS M7000 Imaging System.

Sort decisions for the 1536-well microplates were configured to deposit one target event into specified rows using advanced sorting mode. Sorts were repeated using 1536-well glass-bottom microplates to assess cell occupancy using the EVOS M7000 Imaging System.

As previously described, the prepared CHO cell 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 cell population in the lower left quarter of the plot. A polygon region was used to define the dense portion of the CHO cell population on FSC and SSC signals, and doublets were excluded through gating on an FSC-A x FSC-H plot (Figure 1). Live cells, stained with Hoechst 33342 dye, were gated on a 349 nm 434/17-A x SSC-A plot. All sorts were performed at 150 events/second using single sort mode with default values for drop spacing (value = 32) and a polarity flip volume (value = 0.13 mL).

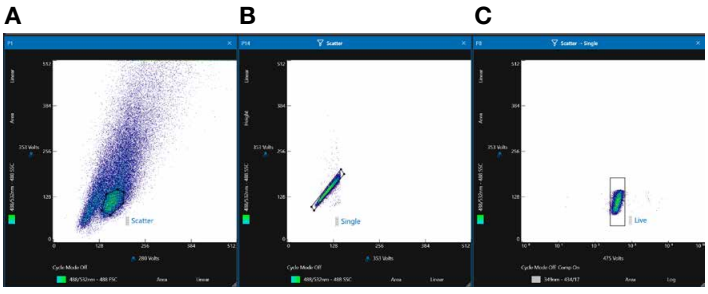


Figure 1. CHO cell plots. (A) A representative forward scatter (FSC-A) versus side scatter (SSC-A) density plot, **(B)** a representative doublet discrimination density plot (FSC-A x FSC-H), and **(C)** a representative live cell density plot (349 nm 434/17 x SSC-A). The sort gate was defined by a region in the FSC-A x SSC-A plot to identify the dense portion of the CHO cell population, a region in the FSC-A x FSC-H plot to define single cells, and a region in the 349 nm 434/17-A x SSC-A plot to define live cells.

Droplet spacing settings reduce stream fanning during sorts by aborting sort events that fall within the defined drop spacing limit so that charged droplets in close proximity do not interact with each other. The polarity flip feature is utilized to reduce the accumulated charge in sort collection vessels by reversing the polarity of the sort streams at the designated interval. When using single sort mode, the sort is only executed if exactly one event is located within the center 50% of the target drop and no particles of any kind are present in the adjacent quarters of both the target drop and the leading and trailing drops. Sorts were performed using straight-down sorting as well as the unique multi-stream plate sorting feature of the Bigfoot Spectral Cell Sorter: four side streams were utilized for 96-well plate sorting (Figure 2) and 384-well plate sorting (Figure 3). This multi-stream strategy allows completion of a plate with only two passes across the plate for 96-well plate sorting and four passes across the plate for 384-well plate sorting, 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. Five 96-well and five 384-well plates were sorted utilizing straight-down sorting, with an additional five plates of each type sorted utilizing four-way sorting. A total of five 1536-well microplates were sorted using only straight-down sorting (Figure 4). Replicated sorts for all plate types were performed using the Infinisort feature. After a sort has finished, Infinisort pauses sample flow and prompts the user to replace the completed plate with an empty plate before repeating the same sort conditions. This process can be repeated until the user stops Infinisort.

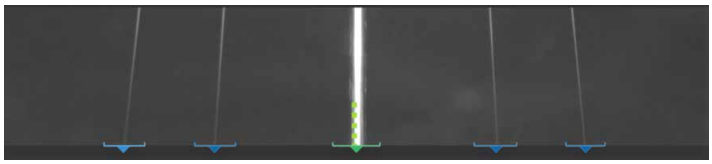


Figure 2. Test pattern with alignment targets of four-way plate sorting mode for 96-well plates.

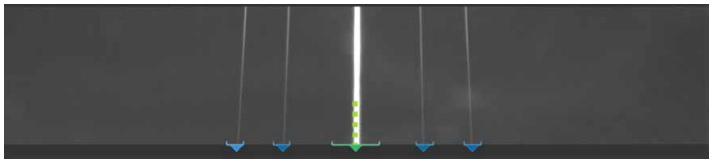


Figure 3. Test pattern with alignment targets of four-way plate sorting mode for 384-well plates.

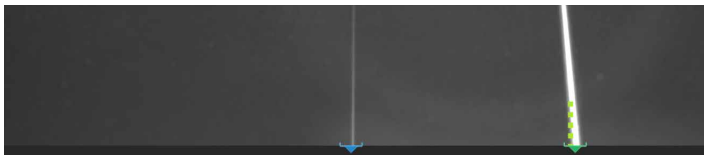
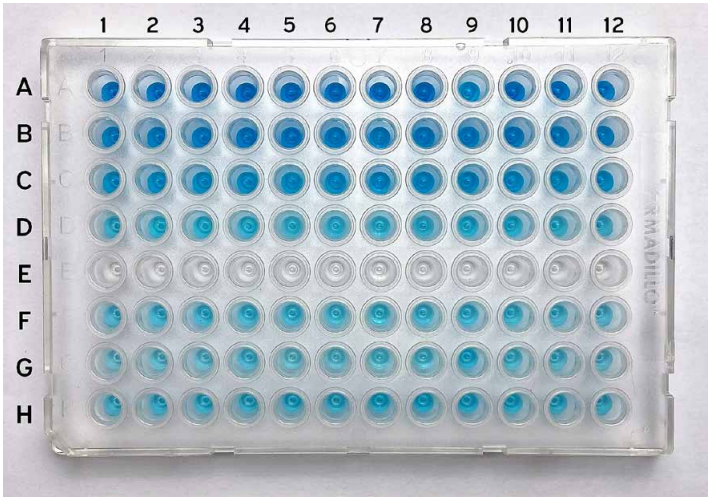


Figure 4. Test pattern with the alignment target of straight-down sorting mode for 96-well, 384-well, and 1536-well plates.

There was no realignment between plates. All plates produced similar results.

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 5 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.



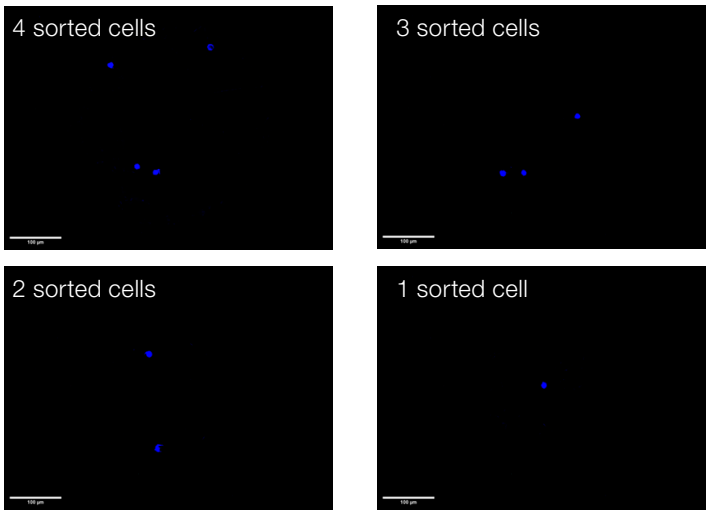


Figure 5. 96-well PCR plate following multi-droplet event sorting suspended in HRP solution along with representative images of CHO cell occupancy in a 96-well cell culture–treated plate using the EVOS M7000 Imaging System. TMB substrate turned blue after sorting and 30 min of incubation in the dark, and cells were imaged using the Invitrogen™ EVOS™ Light Cube, DAPI (357/447 nm), to visualize Hoechst 33342 staining.

Rows F, G, and H were all assigned one target event in the sort logic and contain a similar pale blue color to that of row D.

The elapsed time to complete each 96-well plate was measured as the time the sort was executed in the software until the plate was back in its original position for user retrieval. Tables 1 and 2 show the recorded sort times across all 96-well plates using four-way sorting and straight-down sorting, respectively, as described in the methods section. The average sort time was less than 8 seconds/plate for four-way sorting and less than 20 seconds/plate for straight-down sorting. 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 38.24 seconds for four-way sorting and 99.37 seconds (1.66 minutes) for straight-down sorting. Cell occupancy and sort counts were confirmed using the EVOS M7000 Imaging System (Figure 5).

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

Plate	Elapsed sort time (seconds)
Plate 1	8.06
Plate 2	8.58
Plate 3	7.18
Plate 4	6.96
Plate 5	7.46
Average	**7.65**

Table 2. Elapsed sort time to complete a full 96-well plate using single-droplet straight-down sorting.

Plate	Elapsed sort time (seconds)
Plate 1	19.88
Plate 2	19.89
Plate 3	19.97
Plate 4	19.71
Plate 5	19.92
Average	**19.87**

Figure 6 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 by 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.

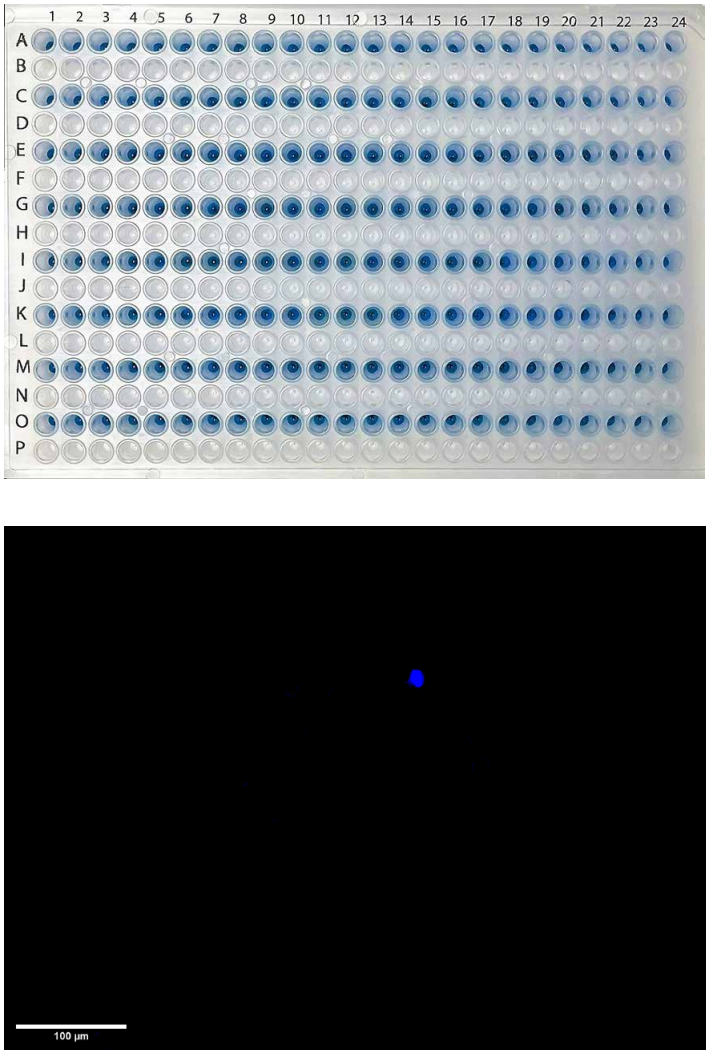


Figure 6. 384-well plate following single-droplet event sorting in HRP solution in alternate rows along with a representative image of CHO cell occupancy in a 384-well cell culture–treated plate using the EVOS M7000 Imaging System. TMB substrate turned blue after sorting and 15 minutes of incubation in the dark. Cells were imaged using the EVOS Light Cube, DAPI (357/447 nm) to visualize Hoechst 33342 staining.

Similar to the 96-well plates, the elapsed time to complete each 384-well plate was measured as the time the sort was executed in the software until the plate was back in its original position for user retrieval. Tables 3 and 4 show the recorded sort times across all plates using four-way sorting and straight-down sorting, respectively, as described in the methods section. The average sort time was less than 18 seconds per plate for four-way sorting and less than 62 seconds per plate for straight-down sorting. All 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 86.91 seconds (1.45 minutes) for four-way sorting and 305.63 seconds (5.09 minutes) for straight-down sorting. Cell occupancy and sort counts were confirmed using the EVOS M7000 Imaging System (Figure 6).

Table 3. Elapsed sort time to complete a 384-well plate using single-droplet four-way sorting.

Plate	Elapsed sort time (seconds)
Plate 1	16.10
Plate 2	18.08
Plate 3	17.15
Plate 4	17.35
Plate 5	18.23
Average	**17.38**

Table 4. Elapsed sort time to complete a 384-well plate using single-droplet straight-down sorting.

Plate	Elapsed sort time (seconds)
Plate 1	59.94
Plate 2	60.00
Plate 3	58.93
Plate 4	64.91
Plate 5	61.85
Average	**61.13**

Figure 7 shows an example of one 1536-well microplate with single droplets sorted into each well.

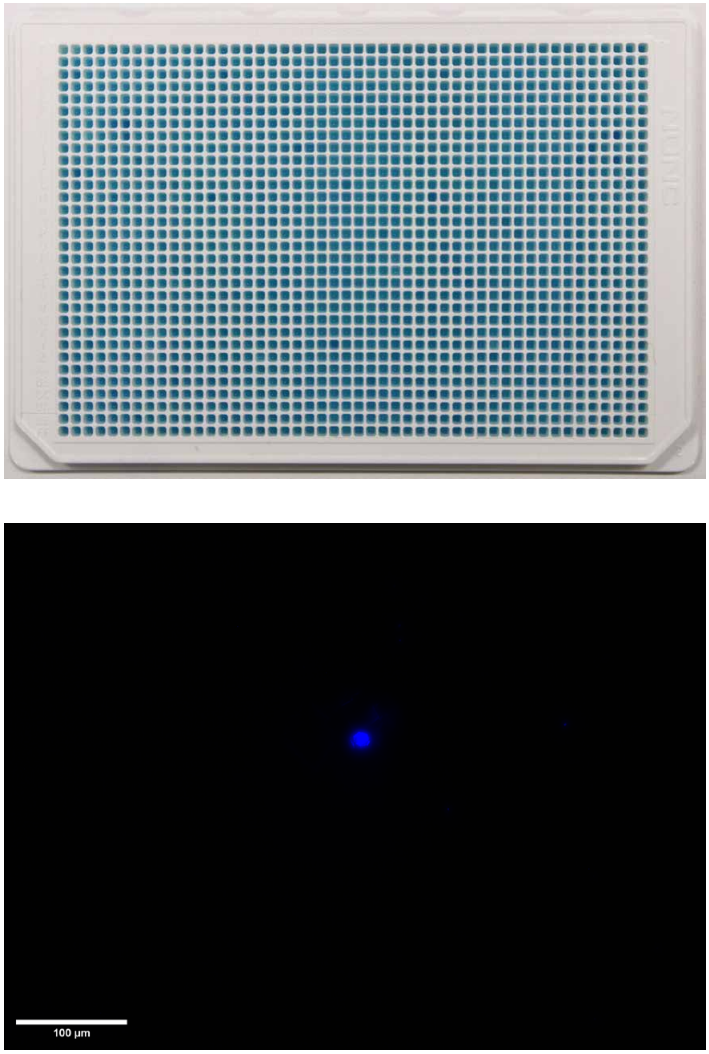


Figure 7. 1536-well plate following single-droplet event sorting in HRP solution in a full plate along with a representative image of CHO cell occupancy in a 1536-well glass-bottom plate using the EVOS M7000 Imaging System. TMB substrate turned blue after sorting and 15 minutes of incubation in the dark. Cells were imaged using the EVOS Light Cube, DAPI (357/447 nm), to visualize Hoechst 33342 staining.

Figure 8 shows an example of one 1536-well microplate with single droplets sorted into specific wells arranged in a "BIGFOOT" text pattern using advanced sorting mode. Both Figure 7 and Figure 8 show the reacted TMB after 15 minutes of incubation. Similar to the 96-well and 384-well plates, the time to complete each 1536-well microplate was measured as the time the sort was executed in the software until the plate was back in its original position for user retrieval. Table 5 shows the recorded sort times across all plates using straight-down sorting into each well. Table 6 shows the recorded sort times across all plates using straight-down sorting into specific wells using advanced sorting to create the "BIGFOOT" text pattern. The average sort time was less than 3 minutes 17 seconds per plate for straight-down sorting into each well, and less than 44 seconds per plate for straight-down sorting into the "BIGFOOT" text pattern.

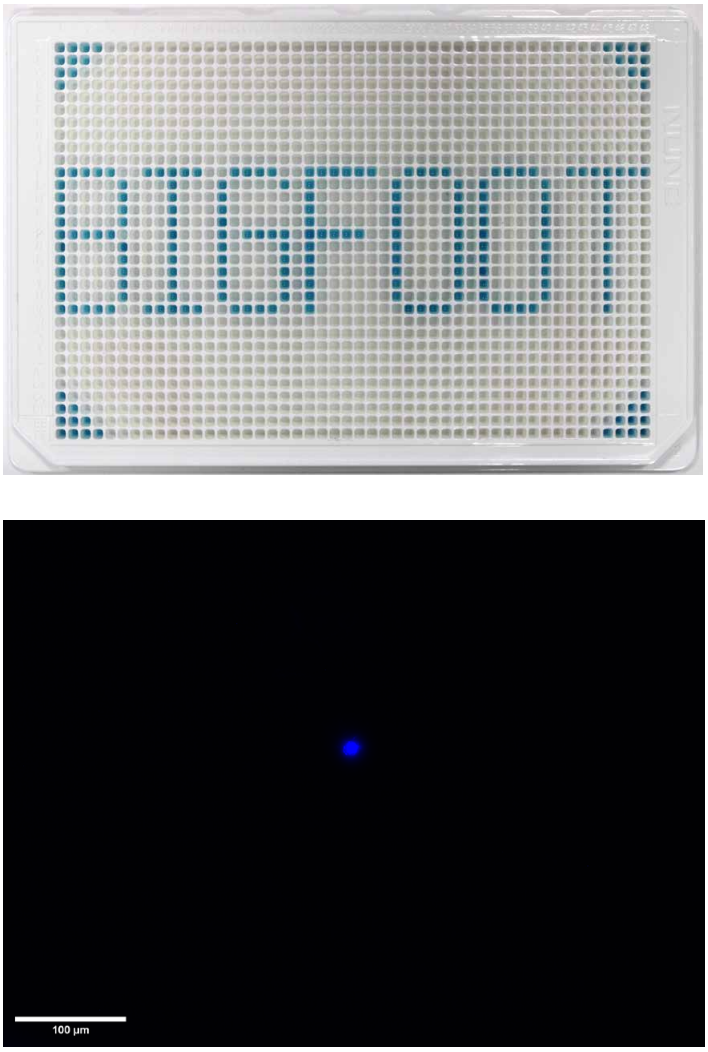


Figure 8. 1536-well microplate following single-droplet event sorting in HRP solution in a "BIGFOOT" text pattern with a representative image of CHO cell occupancy in a 1536-well glass-bottom microplate using the EVOS M7000 Imaging System. TMB substrate turned blue after sorting and 15 minutes of incubation in the dark. Cells were imaged using the EVOS Light Cube, DAPI (357/447 nm) to visualize Hoechst 33342 staining.

All 1536-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 972.13 seconds (16.20 minutes) for full plate sorting and 219.14 seconds (3.65 minutes) for advanced sorting of the text pattern. Cell occupancy and sort counts were confirmed using the EVOS M7000 Imaging System (Figures 7 and 8).

Table 5. Elapsed sort time to complete a full 1536-well plate using single-droplet straight-down sorting.

Plate	Elapsed sort time (seconds/minutes)
Plate 1	196.44/3:16.44
Plate 2	194.13/3:14.13
Plate 3	194.36/3:14.36
Plate 4	194.57/3:14.57
Plate 5	192.63/3:12.63
Average	**194.43/3:14.43**

Table 6. Elapsed sort time to complete an advanced pattern 1536-well plate using single-droplet straight-down sorting into the "BIGFOOT" text pattern.

Plate	Elapsed sort time (seconds)
Plate 1	45.00
Plate 2	43.81
Plate 3	42.31
Plate 4	42.79
Plate 5	45.23
Average	**43.83**

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-size 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.

Therefore, 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 1536-well microplate. Furthermore, the device was designed to travel the entire length and width of a multi-well plate in 0.5 seconds, with minimal lag during transition between wells. Both the positional accuracy and high speed of the plate sorter are the result 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 established 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 because of the tendency of droplets to combine by charge attraction; therefore, minimizing charge and maximizing the deflection distance facilitates 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 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 from the colorimetric conversion of TMB substrate with HRP, it was shown that a single droplet can be sorted in small volumes into 96-well PCR plates, 384-well PCR plates, and 1536-well microplates with 100% targeting accuracy.

Furthermore, it was demonstrated that plates can be sorted with unprecedented speed at averages of less than 8 seconds for 96-well plates and less than 18 seconds for 384-well plates using four-way sorting. High speed is accomplished by the unique multi-way plate sorting capability of the Bigfoot Spectral Cell Sorter, which is available on no other instrument. This feature facilitates the generation of four-sort streams for 96-well and 384-well plates, 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 1536-well microplates. 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 because of its precision, accuracy, robustness, and speed. Furthermore, the usability of the automation feature 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, can enhance the workflows of many applications, and is an asset to research laboratories now and in the future.

References

1. Rodrigues OR, 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, 2023, Products, viewed June 30, 2023, **https://www.10xgenomics.com/products**.

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