Bigfoot Spectral Cell Sorter: cumulative innovations provide rapid, accurate, and precise plate sorting results

Introduction

Sorting cells into multi-well plates is an essential task for applications such as single-cell genomics, cell cloning, and limiting-dilution growth experiments. However, flow cytometric single-cell plate deposition experiments conducted with traditional sorters often have problems that make plate sorting tedious and long in duration, and unproductive. The Invitrogen™ Bigfoot Spectral Cell Sorter is designed with innovative cell-sorting mechanisms to address these issues. Improvements to plate deposition enable unprecedented accuracy, recovery, and speed for single-cell sorting. These include built-in stream calibration and drop delay, built-in media detection imaging that assists with accurate plate setup and verification, well-designed hardware for precise single-droplet targeting, and straight-down sorting for maximum deposition accuracy. In addition, the robust hardware for sort output facilitates maximum flexibility, allowing deposition into 96-, 384-, 1,536-well, or other standard and nonstandard plates. Finally, and most impressively, the Bigfoot Spectral Cell Sorter is capable of 4-way sorting into 96-well plates and 8-way sorting into 384-well plates for unprecedented speed. This multi-population plate sorting feature is not available on any other cell sorter.

To fully appreciate the capabilities of the Bigfoot Spectral Cell Sorter, it is worth noting that traditional plate sorting platforms produce only a single side stream, which restricts plate sorting to one target population at a time. The efficiency, or recovery, of the sort (defined as the collected cell targets divided by the available cell targets) on a legacy sorter is always much lower on plates than the recovery of the same sample sorted into tubes. When a traditional plate holder is repositioned for each well, valuable time is wasted that could otherwise be used to recover additional targets that are diverted to waste. Furthermore, if several unique cell populations are required for an experiment, the traditional sorter discards additional target cells into the waste stream while it sorts the first population of interest. Sort speed, deposition accuracy, and consistency over time are considerably different among manufacturers, and recalibration between plates is necessary for some traditional sorters. In contrast, the Bigfoot Spectral Cell Sorter offers extremely robust and high-performance plate sorting features that provide consistent deposition efficiency and high-speed cell recovery for optimal input into downstream experiments.

Instrument optimization for plate sorting

Accurate drop delay is crucial for purity and recovery

The drop delay setting on a cell sorter is a mechanism that measures the time necessary for a target cell to reach the droplet breakoff point in the sample stream so the droplet can be charged and directed to the proper collection receptacle. A correct drop delay setting is essential for optimal cell sorting. Conversely, an incorrect drop delay value results in the loss of the target cell and sometimes inadvertently allows the collection of unwanted cells, causing suboptimal sort purity and recovery. As shown in Figure 1, even very small errors in drop delay affect cell recovery more than purity. This difference can be attributed to the preponderance of empty droplets in the droplet queue.

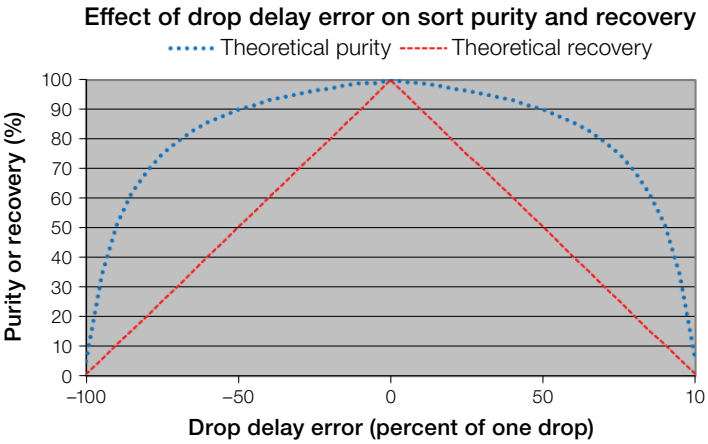

Figure 1. Theoretical recovery and purity when sorting a 5% target population in single-cell mode. Target recovery is directly related to the magnitude of the drop delay error, while purity is not affected until the magnitude of the error is very large. Sort purity will degrade for samples with lower percentages of targets, and will increase for samples with higher percentages of targets. Low sort sample quality, especially of samples with many cell aggregates, will also show degraded purity results. Adapted from Durack G [1].

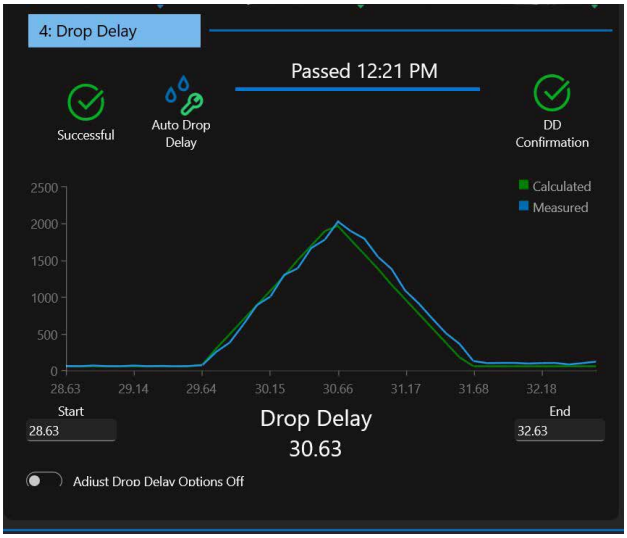

Figure 2. Determination of drop delay setting. The Bigfoot Spectral Cell Sorter automatically sets drop delay at the point where the maximum number of beads is detected and collected in the dedicated port. The drop delay setting in which the highest number of beads is detected is used to construct a triangular function similar to the theoretical recovery plot in Figure 1. This triangular function is then compared to the original drop delay reading curve to find the maximum correlation point, which is used as the drop delay setting. A Pearson correlation coefficient is computed to measure how closely the measured curve matches the computed curve. A coefficient greater than 0.8 provides acceptable confidence.

For example, when the droplet generation frequency is set at 96 kHz and the event rate is 9,600 cells per second, only 10% of the droplets will contain a cell. Thus, sorting an empty droplet instead of the correct droplet containing the target cell will not affect purity as greatly as it will affect recovery. The plot of theoretical recovery and purity in Figure 1 highlights the amount of recovery and purity loss as drop delay errors increase from the center of the correct breakoff droplet point.

Automatic determination of drop delay

To determine the ideal drop delay setting, the Bigfoot Spectral Cell Sorter uses a novel method to cycle through a series of whole droplets and fractions of droplets to determine the setting that returns the highest recovery of target beads. Target drop delay beads are deflected into a dedicated port within the multi-position waste catcher. A 405 nm laser and a silicon photomultiplier (SiPM) act as a mini-cytometer to enumerate the beads while drop delay settings are cycled to test droplet charge timing. The drop delay on the Bigfoot Spectral Cell Sorter is automatically set at the point where the maximum number of beads is collected in the dedicated port. This method is used to produce the plot shown in Figure 2.

Manual drop delay verification wizard

While the correct drop delay is always necessary for sort experiments, it is critical for plate sorting where accurate cell event numbers are desired for each well.

Even though the drop delay determination of the Bigfoot Spectral Cell Sorter is one of the best methods available, confirmation using an alternative method is sometimes desired. For many researchers, the gold standard for drop delay verification is the microscope slide method, where brightly fluorescent beads are sorted onto a microscope slide. Puddles of sorted beads are deposited on the slide as the instrument runs through a series of drop delay settings. The investigator then manually views the slide under a microscope and counts the beads in each puddle. Invitrogen™ Sasquatch Software for the Bigfoot Spectral Cell Sorter includes a slide wizard that assists with this method of verifying the drop delay. Using the wizard, three puddles of beads are sorted onto the slide using the previous automatically determined delay setting and two other settings that include plus one and minus one droplet from the optimal setting. If 100 beads are sorted, the two puddles representing the plus one setting and the minus one setting should not contain beads, while the center puddle should contain 100 beads. It is only necessary to count the number of beads in the side puddles. If beads are found in a side puddle, the investigator can input the puddle number and count of errant beads into the wizard to correct the drop delay setting. As an example, an error of 3 beads on the puddle representing the lower drop delay setting would result in a –3% correction to the drop delay.

Large or irregularly shaped target cells can disrupt droplet formation, resulting in variation of the droplet breakoff point as described by Arnold and Lannigan [2]. Therefore, the drop delay wizard can also be used to confirm the drop delay using the actual target cells from the sort sample.

Rmax verification compatible

Another option for checking drop delay accuracy is the Rmax method [3], which requires collection of the waste stream to determine the percentage of target cells that were diverted to waste.

Multi-way plate sorting

The Bigfoot Spectral Cell Sorter uses a unique multi-way sorting method for plate sorting (Figure 3). When sorting into 96-well plates, four side streams are used. Similarly, eight side streams are used for 384-well plates. Multi-way sorting is used by default, although a more traditional single-stream process can also be selected.

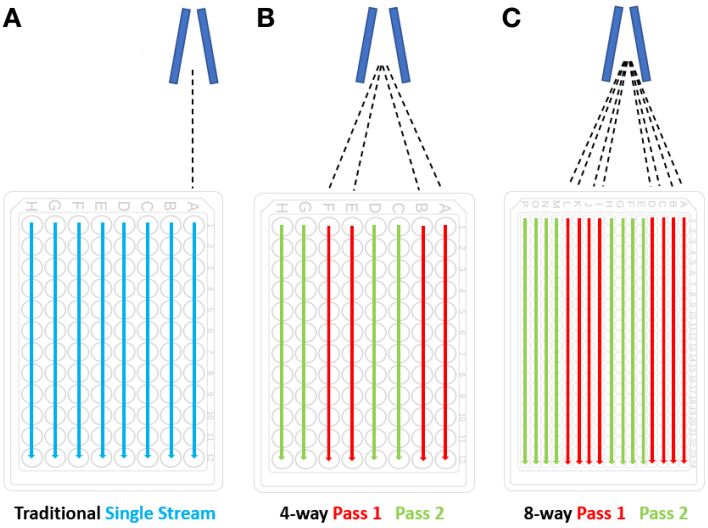

Figure 3. Multi-way plate sorting compared to traditional plate sorting. (A) Traditional plate sorters deposit targets into each well individually, moving stepwise from well to well, thus stopping the plate movement 96 times for a 96-well plate. **(B)** The Bigfoot Spectral Cell Sorter uses 4-way sorting to make two passes across a 96-well plate, thus stopping the plate only 24 times for a four-fold reduction in plate positioning time. **(C)** The Bigfoot Spectral Cell Sorter uses 8-way sorting for 384-well plates, making two passes across the plate and stopping 48 times for an eight-fold reduction in plate positioning time.

Multi-way sorting reduces the number of plate-positioning movements, thus decreasing the required time to complete a plate sort. Optionally, traditional movement can be selected by choosing the straight-down sorting option. This option deflects the waste stream to the side, rather than the sorted droplets, and can be helpful when sorting into very small amounts of collection buffer. The straight-down option is also the correct choice for 1,536-well plates.

It is important to note that these two-pass sorts require careful planning when sorting several different target populations into the same plate. The speed of each pass will be determined by the rarest target population, and very rare populations could eliminate the collection of more abundant targets if all the very rare targets are included in the first pass, with other targets assigned only to the second pass. Careful consideration of experimental goals is required when sorting multiple target populations.

Plate calibration

Positioning the plate to catch sorted droplets in the center of the assigned wells is essential for plate sorting. Mispositioning of a sorted droplet usually comes from two sources: the sort stream not being correctly aligned, and the plate mover drifting from its set position. The Bigfoot Spectral Cell Sorter is designed to eliminate these problems. The side streams for a defined sort medium are automatically positioned using set crosshairs determined when the plate is defined (Figure 4). This ensures that the side streams are correctly positioned independently of nozzle size or other local environmental factors such as heat and humidity.

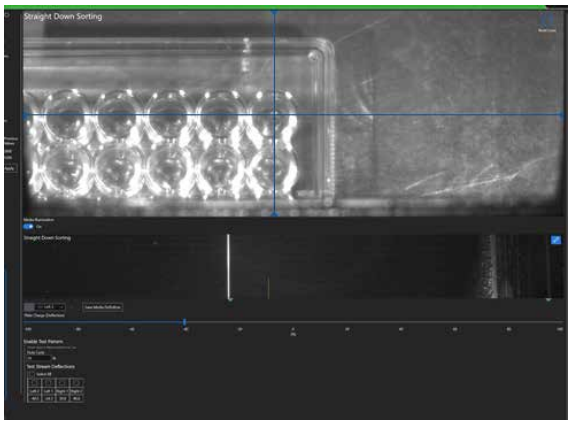

Figure 4. Plate positioning for sorted droplets. Plate calibration is accomplished using an LED camera to visualize test droplets sorted onto the plate cover. A software crosshair tool aids in identification of the correct plate position. Plate positioning of the Bigfoot Spectral Cell Sorter has been found to be very stable within sorting experiments and in day-to-day setup. As with drop delay, plate aiming using the sort target cells may be necessary for large or unusually shaped cells.

The deflection plates, droplet formation, and plate movement of the Bigfoot Spectral Cell Sorter were designed to ensure the accurate deposition of droplets within 10% of the surface area of the 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 are the product of a custom-designed linear actuator system, fine-pitch lead screws, high-torque motors, and 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 the 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 facilitate highly stable and accurate deposition [4].

Plate sort verification

Kissner et al. previously reported on droplet deposition accuracy of the Bigfoot Spectral Cell Sorter using the HRP/TMB method [4]. While the colorimetric HRP reaction can signal whether a droplet was deposited into a well, it cannot indicate whether that droplet contained a target particle. Therefore, to further test the speed and accuracy of plate sorting, mouse splenocytes were sorted and detected using a PCR-based assay. Figure 5 shows the results of several test plate sorts.

The Bigfoot Spectral Cell Sorter sorted cells into a 96-well plate using the traditional single-stream method in 22 seconds, whereas the fastest alternative cell sorter on the market (MoFlo™ cell sorter) required 50 seconds for a similar sort. Using 4-way sorting, the Bigfoot Spectral Cell Sorter reduced the time required to as little as 7.2 seconds. The traditional method required 63 seconds for a 384-well plate, while 8-way sorting required as little as 12 seconds.

Advanced sorting, Infinisort feature, and index sorting

The advanced sort options of the Bigfoot Spectral Cell Sorter allow the researcher to select different gates, sort counts, or sort modes for each well of the plate, providing optimal flexibility for complex sorting experiments.

The Infinisort feature is particularly helpful when sorting into multiple plates. Within the biosafety containment area, the Infinisort feature pauses sample flow at the end of each plate sort and then opens the sort output chamber for rapid plate removal and insertion. The user can then resume the sort from within the biosafety containment area without additional risk to the sample or operator safety.

Index sorting is always enabled for sorts on the Bigfoot Spectral Cell Sorter. All saved FCS files for sort experiments include the parameters and destination media for both sorted and aborted events. Indexed sort data are useful for troubleshooting sorting experiments or correlating sort data with downstream analysis such as genomic sequencing, cell cloning, or limiting-dilution growth assays.

A

Plate type	Stream	Time (sec)	Bands detected/ expected
96-well	4-way	7.7	88/88 (100%)
96-well	4-way	7.2	87/88 (97%)
96-well	Single	22	85/88 (97%)
384-well	8-way	12	341/352 (97%)
384-well	8-way	12.5	338/352 (96%)
384-well	Single	63	346/352 (98%)

B

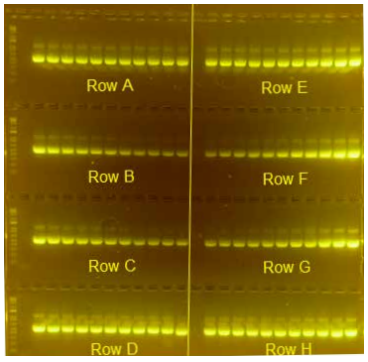

Figure 5. Evaluation of the speed and accuracy of plate sorting. Freshly isolated mouse splenocytes were stained with Invitrogen™ SYTOX™ Blue stain and sorted in single-cell mode gated on scatter and SYTOX Blue stain into 96-well and 384-well PCR plates, followed by PCR and gel analysis. The single-way and multi-way stream sort methods were compared for speed and accuracy. **(A)** Speed and accuracy results of the Bigfoot Spectral Cell Sorter. **(B)** Representative gel showing PCR products from cells after a 96-well 4-way sort. The first column of the plate was left empty as a negative control. Data courtesy of Columbia Stem Cell Initiative Flow Cytometry Core Facility, Columbia University Irving Medical Center.

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Discussion

Plate sorting requires the accurate deposition of the target cells into the correct well of the catch plates. Using a unique and robust drop delay setting method to identify the droplet containing the target, and an accurate and consistently positioned catch plate, the Bigfoot Spectral Cell Sorter yields exceptional plate sorting efficiency. The precision high-speed plate holder stepping motors and the revolutionary 4-way and 8-way plate sorting methodology produce unparalleled sort speeds. Index sorting by default, rapid plate exchange using the Infinisort feature, and unrestricted sort options for multiple target cells make the Bigfoot Spectral Cell Sorter the market leader in cell sorting.

References

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