



Single-Cell Sorting for Clonal Enrichment

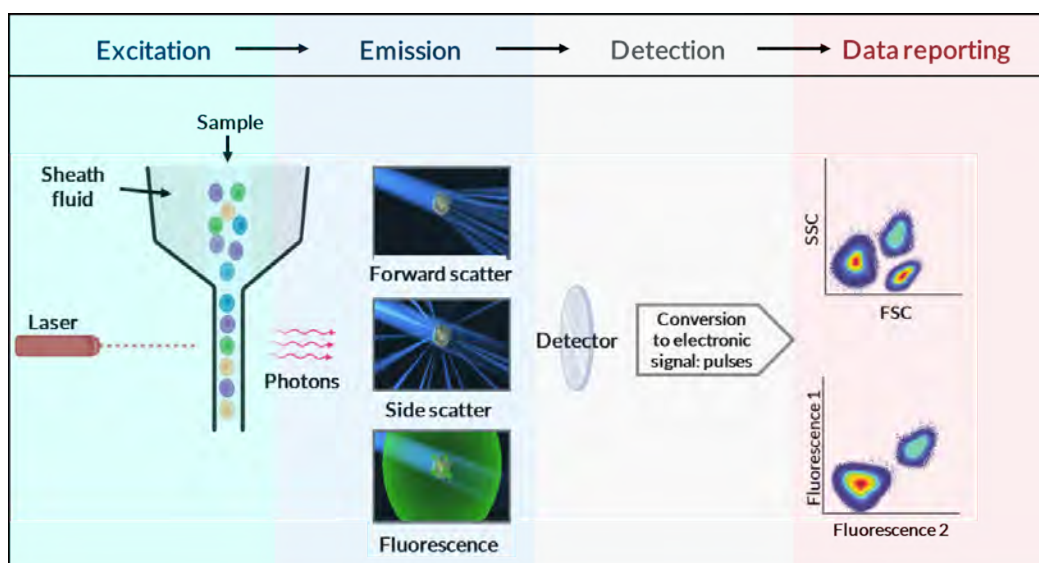
Single-Cell Sorting for Clonal Enrichment

A highly useful utility of cell sorters is the capability to sort single cells into multiwell plate formats. In recent years, advances in single-cell genomics and transcriptomics have increased the use of single-cell deposition experiments.

These techniques can also be quite useful in synthetic biology workflows, as they allow for the enrichment of target populations and deposition of accurate numbers into multiwell formats to ease screening for ideal clones and culture conditions. Deposition and enrichment can be based on fluorescent reporters such as green fluorescent protein (GFP) or red fluorescent protein (RFP), or simply based on light scatter.

In this application note, focus will be placed on how to set up a single-cell sorting experiment with a plate output, the use of the 0.5 drop and 1 drop envelopes, and the possible outcomes for experiments using these envelopes. Example data of a fluorescent bacterium being sorted and visualized is also shown.

All flow cytometers and flow-based sorters have some things in common. Particles are measured in a liquid suspension (flow), particles are driven in front of a laser source (excitation), particles emit light based on fluorescent labels or scatter laser light (emission). This emitted light is captured by detectors, amplified, and converted into a digital value the operator views in the software.



Once data is visualized, regions can be drawn on the data to exclude particles that are not of interest from further analysis. With a sorter, these regions are also used to narrow down the particles to be sorted to include only the desired targets. When a region is drawn it can be used to limit the data shown on another data plot; this process is known as gating.

Setting up for a single-cell sort

1) Creating regions, gates and sort logic

All sorting experiments begin with analyzing sample data. In this case, the data are simple. HeLa cells transfected with GFP were harvested and a small amount was run on the CytoFLEX SRT benchtop sorter.

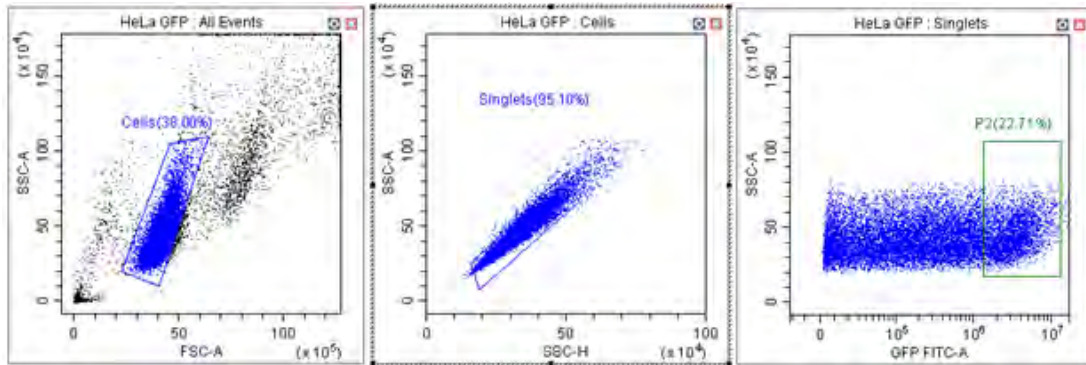


Figure 1. Example data for single-cell sorting.

For this experiment it is possible to get the desired events with 3 plots and 3 regions. In this experiment, the desired events are the top 20% GFP-expressing cells. Three gates were used to ultimately define this population. First, cells are separated from debris by looking at FSC-A vs SSC-A. FSC is forward scattered laser light and refers to light scattered as the particle passes through the laser and detected 180° from the laser. It is impacted mostly by particle size, but is also affected by particle composition. SSC is side scattered light. This is quite similar to FSC but is collected at a 90° angle from the laser. It is primarily impacted by particle composition, but particle size is also a factor in SSC signal. As particle size falls below 1 micron, SSC is often more sensitive to detect and separate particles of different sizes. For different cell types, it may be necessary to increase the gain for these channels to effectively resolve live cells from small debris or dead cells. At low gain settings, small particles might be lost in the electronic noise of the instrument, while at high gain settings, electronic noise is amplified, resulting in high data rates and difficulty seeing events caused by particles of interest.

For all sorting experiments it is important to exclude doublets (cells adhering to one another) from sort logic. There are several means to accomplish this, and in this case a plot of SSC-A (area of the pulse measured) vs SSC-H (height of the pulse measured) is used. With this method, singlets will have a very tight association between these 2 parameters. Any doublets will have a similar Height signal, but due to the second event sticking to the first, they will have increased Area signal. In analysis experiments this prevents mistaking cell identity due to 2 cells being stuck together. In sorting, this prevents sorting an undesired cell stuck to a desired cell, or sorting 2 cells where only 1 is required.

Finally, in this experiment, the decision was made to sort only the highest expressing GFP-positive events. A region was drawn to include the top expressors of the fluorescent protein. Finally, the highest GFP-expressing events were included in gate P3. Using gate statistics, the region was drawn to include the top 20% expressors of the fluorescent protein.

2) Setting up the plate sort

The CytoFLEX SRT sorter's automated setup workflows have already done the work of finding stable droplet formation and drop delay. The automated sort calibration workflow finds a droplet generation frequency (how many vibrations, or drops are formed in a second) and amplitude (vibration intensity), which results in stable droplet formation to allow for predictable drop delay (the wait time between particle detection and stream charging). The user calibrates the plate positioning prior to defining the logic and plate layout (for more information see the CytoFLEX SRT IFU, Chapter 5).

Create a new tube for your experiment and click the plate sorting icon.



The following screen appears:

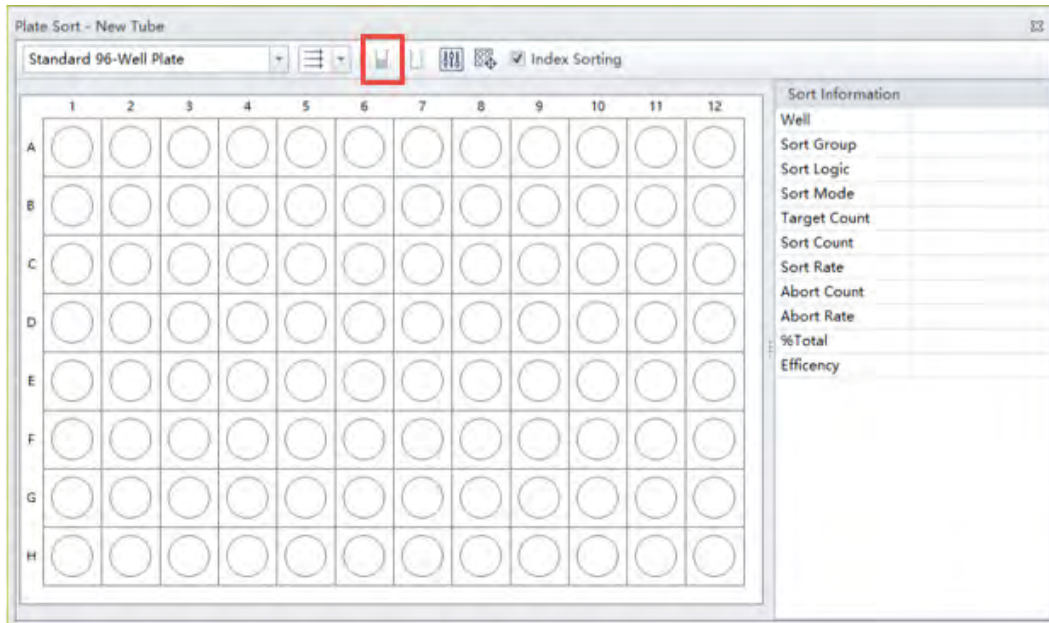


Figure 2. A blank plate layout. Outlined in red is the button used to define a well or series of wells as a sample well.

First, indicate the media (plate type) you are sorting into. Then indicate the direction in which you want the plate to be filled, whether by rows from left to right, across rows, down columns, or serpentine across columns or rows.

Select all the wells you wish to have cells deposited into. In this case we have left the outer wells empty. This is often done in cell culture experiments, as the outer wells can dry out quickly over long-term culture. Any wells can be left open or filled as the experiment requires.



Figure 2. A plate layout showing wells assigned to be sorted.

Name the Sort Group (free text) and assign well color to the group. In this case, we named it **GFP+** and assigned it a green color. Define the population to be sorted into the indicated wells by choosing from the hierarchical gate list, choosing **Single** Sort Mode and assigning the desired Target Count. Click OK to accept.

Once **Start Sort** is clicked, plate sorting begins. Multiple plates can be run with the same settings by choosing Duplicate tube without data and clicking **Start Sort** again. Additional populations can be sorted into empty wells by defining individual wells or groups of wells. However, only one gate can be assigned per well.

3) Sort Envelopes & Sort Mode

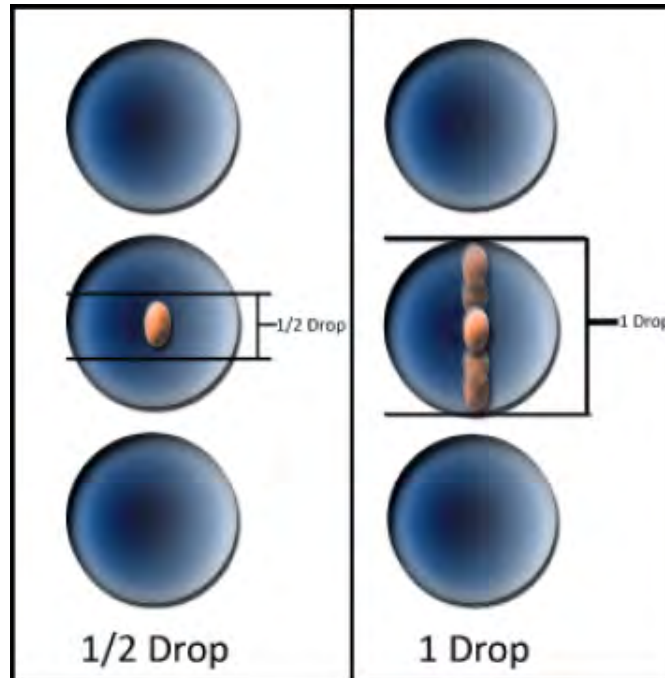


Figure 3. An illustration of 0.5 drop and 1 drop sort envelopes, indicating locations in the droplet that would be sorted in each envelope condition.

When sorting into plates for single-cell applications, an important consideration is the sort envelope size, which considers the position of the cell within the droplet to determine whether to sort or abort the event. It asks, “Will the event remain in the desired drop, or will it move to the preceding or trailing droplet?”

In Figure 4 above, the effect of two envelopes used with Single sort mode are illustrated. A 1 drop envelope indicates that as long as the event of interest is predicted to be anywhere within the droplet, that droplet will be sorted and there are no contaminant cells in the leading and trailing edges of the drops above and below. However, before this event was in a droplet it was in the flow cell for interrogation, and then in a stream in the air. As the interface between droplets narrows and droplets begin to break off, cells that are in the narrowing spaces might move into a trailing or preceding droplet--dependent upon their position in the stream--possibly resulting in sorting an empty droplet into a well if the event moves out of the charged droplet. If the target cell is not in the predicted drop, sorting the empty drop results in an empty well. The CytoFLEX SRT sorter deals with this issue by using a Guard Band that will abort cells with contaminants that are in the edges of neighboring drops. An illustration of Guard Band can be seen below in Figure 5.

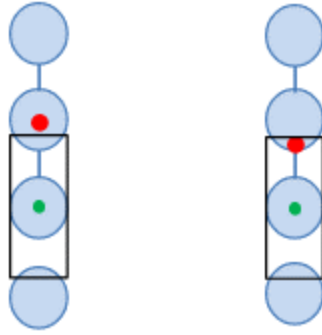


Figure 5. An illustration of sort Guard Band. The black rectangle indicates the 15% of a drop area in the leading and trailing drops that will cause aborts if a contaminant is likely to be present. The left event would be sorted, but the right would be aborted.

The 0.5 drop envelope addresses the issue of nearby contaminants by sorting only droplets where the cell of interest is predicted to be in the center 50% of the droplet. This lowers the chance of sorting an empty droplet into a well. The default sort envelope when using Single mode in the CytoFLEX SRT sorter is 0.5 drop.

In a plate sort, using a 1 drop envelope will have two likely outcomes. First, abort rate will be lower and plates will fill more quickly with less loss of target cells. Second, overall plate filling efficiency could drop as a result of some wells receiving empty droplets. Alternatively, when using the default 0.5 drop envelope the situation is reversed. Plates may fill more slowly, but overall plate filling efficiency is higher. Both plate efficiency and filling speed will also be affected by sample properties such as cell size, event rate of the sort, and target cell percentage.

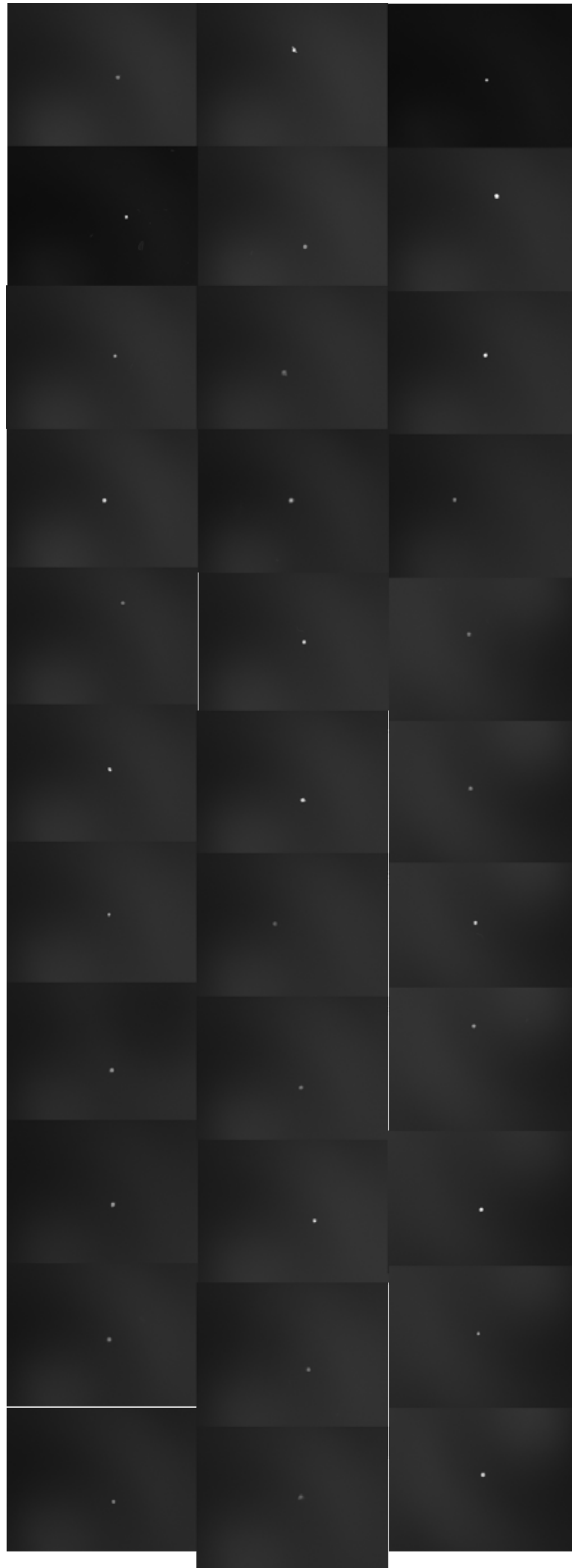


Figure 6. HeLa GFP cells were sorted with sort mode Single 0.5, filling the plate across rows in serpentine fashion. Sort collection was in small wells of media on a glass slide. After allowing the cells to settle, images were taken under low magnification to show single cells in individual spots. Images represent 33 wells from 3 different experimental slides. 1 well per experiment had 10 cells/well (image not shown) to allow for focusing of microscope optics. Left column shows experiment 1, center column experiment 2, and the right column shows experiment 3.

Single Bacterium Sorting (>250 μm)

To show the capability of the CytoFLEX SRT sorter to acquire and sort single bacterium, a sample of GFP-labeled activated *Bacillus subtilis* was analyzed using the FITC (525/40) channel. GFP-positive bacteria were gated and then a single bacterium was sorted onto a slide and verified by fluorescence microscopy. The test was performed to verify the ability of the machine to sort a relatively small biological (2-6 μm in length and 0.25-1.0 μm in diameter) with a refractive index similar to that of an extracellular vesicle at 1.38.

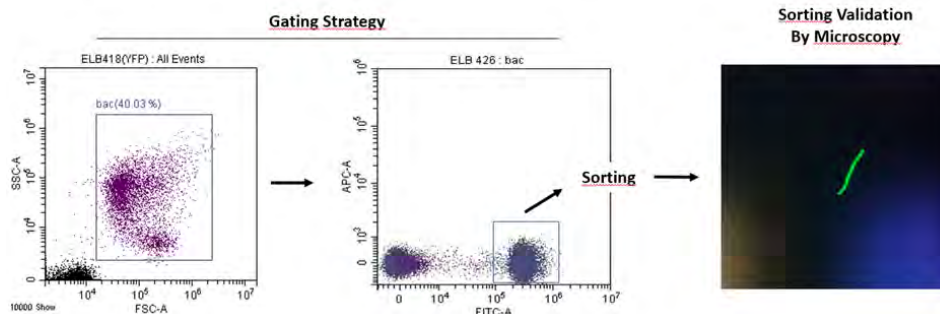


Figure 7. *B. subtilis* test sort. Cultured bacteria were sorted with sort regions to remove debris and doublets, and finally for YFP-positive bacteria (VSSC Threshold 1650/Gain 300, FITC-750, SSC-100). Following deposition on a glass slide the bacterium deposited was visualized using an Olympus BX62 microscope. Images were captured using an Andor Sona camera at 40x objective with immersion oil.

TIPS FOR SUCCESS

- It is important to calibrate the plate to ensure the stream hits the target, especially when adding a new sort media output.
- When filling the plate completely is most important, the default Single mode 0.5 drop envelope is recommended.
- Confirming plate calibration takes little time and can provide reassurance that droplets will hit targeted wells.
- Plate efficiency can be measured in numerous ways, such as PCR confirmation, multiwell plate imaging, or visual confirmation in a fluorescent microscope (shown in Figure 6).
- In a low-volume microplate, be mindful of both plate well and droplet volume. A cell that was sorted may land outside of collection media and desiccate. Also, sheath in the droplet is sorted along with the cell, so when sorting more than one cell per well in low volumes, the sheath contribution to overall volume could become an issue.

Purifying and enriching populations is useful in a variety of workflows, including strain selection and characterization in synthetic biology and microbiome research. The CytoFLEX SRT sorters and CytoFLEX analyzers provide the sensitivity required to detect and enrich these often small and dimly fluorescent cells. Paired with easy-to-learn software and automation integration, a variety of possibilities are available to explore.

Beckman Coulter Life Sciences is your partner in synthetic biology workflows. Please contact your local Beckman Coulter representative for more information.



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2024-GBL-EN-106194-v2

