High-Speed Sorting of Rare Cell Subsets with High Purity and Viability Using the S3e Cell Sorter

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Cell Sorting

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Abstract

Fluorescence-activated cell sorting is a sophisticated way of quickly isolating cell subsets of interest with exquisite precision and specificity. However, isolating rare cells (frequency <1%) accurately and quickly continues to present a challenge for many scientists. This is especially problematic when working with fragile cells, such as primary cells, which lose quality when kept in suboptimal conditions for extended periods. Speeding up the process with higher cell sorting rates, however, can damage cells or lead to poor recovery in some instruments. The ideal device for this application must be able to accurately sort cells at high speeds while preserving viability. This proof-of-concept study demonstrates how the S3e Cell Sorter is uniquely capable of cell isolation with high recovery and viability, even at high speeds.

Introduction

Many research applications call for isolating rare cells from a heterogeneous cell mixture. However, when the cells of interest make up less than 1% of the total population, acquiring the yield and viability needed for downstream studies becomes difficult. For example, a typical mammalian transfection by electroporation requires an input of 1,000,000 cells. If the cells of interest make up 1% of the total population, sorting the required 100,000,000 cells at a typical rate of 5,000 events (cells)/second would take roughly 6 hours (Figure 1). When factoring in the typical 50% recovery associated with many cell sorters, the actual collection time is closer to 12 hours.* These time scales are not ideal for cell viability and can further impact yield when working with fragile cells.

One way to reduce run times is to increase the event rate; but most cell sorting systems are designed to operate within confined speed parameters and lose performance at higher speeds of 10,000 events/second. Some high-speed cell sorting instruments discard a significant number of sorted sells as coincidence events, resulting in poor recovery and poor subsequent yield. Further, high speeds can reduce cell viability in studies involving fragile cells. The S3e Cell Sorter is equipped with unique capabilities that allow isolating rare cells at high speeds of up to 10,000 events/second without sacrificing recovery and viability.

Here, we isolate live, stably transfected GFP+ and RFP+ HEK 293 cells on the S3e Cell Sorter, at very high speeds, to greater than 98% purity. Cell populations with frequencies of 1% and 2.5% demonstrate greater than 80% recovery. Importantly, all cell populations sorted by the S3e Cell Sorter had 99% post-sort viability, establishing the S3e as the ideal instrument for isolating rare cells, gently, even at high speeds.

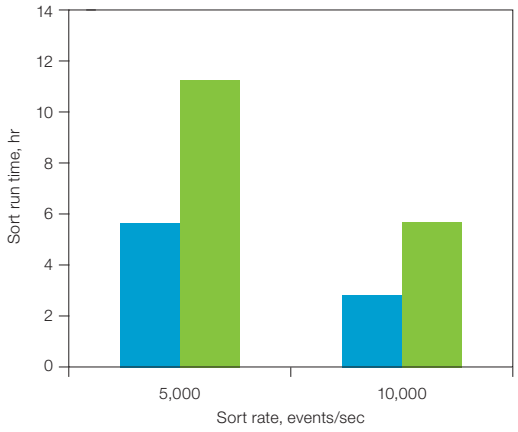

Fig. 1. Run times for sorting 1 x 10^6 cells at 5,000 events/second vs. 10,000 events/second. Indicated run times do not include instrument setup. Percent recovery = (number of sorted cells) / (number of sorted cells as reported by the instrument). 100% recovery (■); 50% recovery (■).

* The indicated times do not account for sample preparation and instrument setup.

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Methods and Results

S3e Cell Sorter

The S3e Cell Sorter (Bio-Rad Laboratories) is a compact, walk-away automated cell sorter with key technologies that make it ideally suited for isolating rare cells. The jet-in-air sorting feature maintains a constant stream velocity for gentle and accurate cell sorting, even at high speeds. Concurrently, the built-in ProDrop technology performs automatic drop-delay calculations, which leads to accurate cell sorting with purity over 99%. Instrument setup and routine calibration steps were performed as directed in the S3e Sorter manual.

Cell Preparation

HEK 293 GFP and RFP (GenTarget) stably transfected cell lines and nontransfected control HEK 293 cells were cultured separately in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich), supplemented with 1% MEM nonessential amino acids, 1% penicillin-streptomycin, and 10% fetal bovine serum (FBS; VWR International).

In preparation for cell-sorting experiments, cells were trypsinized (0.25% trypsin/1x EDTA; VWR), transferred to a 15 ml conical tube and centrifuged at 300 rcf for 5 min. Cells were then washed and resuspended in phosphate buffered saline (PBS). Cell counts and trypan blue viability checks were performed with a TC20 Automated Cell Counter (Bio-Rad). Next, single-color cell suspensions for GFP, RFP, and VivaFix 410/450 Viability Dye and unstained controls were prepared at 1 x 10^6 cells/ml for the compensation matrix calculation. The experimental sort sample was prepared by adding GFP- and RFP-expressing HEK 293 cells to control, non-fluorescent HEK 293 cells at a final concentration of 1% and 2.5%, respectively. The experimental sample was then centrifuged at 300 rcf and resuspended in 1 ml of sort buffer (1x PBS, 1 mM EDTA, 3% FBS) to a final concentration of 1.68 x 10^6 cells/ml. All samples were filtered through a 40 µm filter to avoid cell clumping and kept on ice until cell sorting.

Cell Sorting

Prior to the run, a neutral density filter (included with the S3e Cell Sorter) was placed in front of the FL2 PMT to ensure that the brighter GFP samples remained on scale in the density plots.

Data collection and analysis was performed using ProSort Software. A series of discrimination gates were created on multiple plots for isolating healthy single cells expressing either GFP or RFP. Size discrimination was achieved through FSC vs. SSC; singlets were discriminated by both FSC-H vs. FSC-W and SSC-H vs. SSC-W; live cells were gated at FL1 vs. FSC; and the sort plot was set with gates on FL2-positive (GFP) and FL4-positive (GFP) populations. The HEK 293 negative controls and single-stained samples were then analyzed in cycle mode. Voltage for FSC, SSC, FL1, FL2, and FL4 was adjusted to ensure all events were collected on scale with optimal separation between control and fluorescent samples.

To calculate the required compensation, 20,000 single-color and negative control events were first collected from each sample, and individual FCS files were saved. The Auto Compensation wizard within ProSort Software was run using a universal negative source (HEK 293 negative control cells) and single-stained samples for VivaFix 410/450 (FL1), GFP+ HEK 293 (FL2), and RFP+ HEK 293 (FL4). The compensation matrix was then applied to all relevant plots.

RFP- and GFP-positive populations were then determined with a pre-sort test run. Ten thousand events per second from the mixed cell sort sample were collected on a FL2 vs. FL4 density plot. The positive populations were gated with rectangular sort regions; GFP was selected for right-sort and RFP for left-sort. Sort logic was then set to collect all events in purity mode for both GFP+ and RFP+ cell populations. Sorted cells were collected in polypropylene FACS 5 ml collection tubes that had been pre-coated on their inner surface with FBS and contained 1 ml of standard HEK culture media. The test sample was sorted at approximately 10,000 events/second and subsequently reanalyzed at 1,000 events/second to determine the sort purity and sample recovery.

High-Speed Isolation of Pure, Healthy Cells

Using purity sort mode and the gating strategy shown in Figure 2, we sorted live, healthy GFP+ (29,567) cells and RFP+ (68,121) cells at approximately 10,000 events/second, corresponding to 80% of available GFP and 92% of the available RFP total cells in the final sort gates. We centrifuged and resuspended the sorted GFP+ and RFP+ cells in 0.5 ml of sort buffer and reanalyzed the cells on the S3e Cell Sorter. We measured a 92% recovery at 99% purity for the RFP cell population and an 80% recovery at 98% purity for the GFP cell population. Both cell populations consisted of 99% viable cells when measured with a TC20 Automated Cell Counter prior to analysis (Figure 3).

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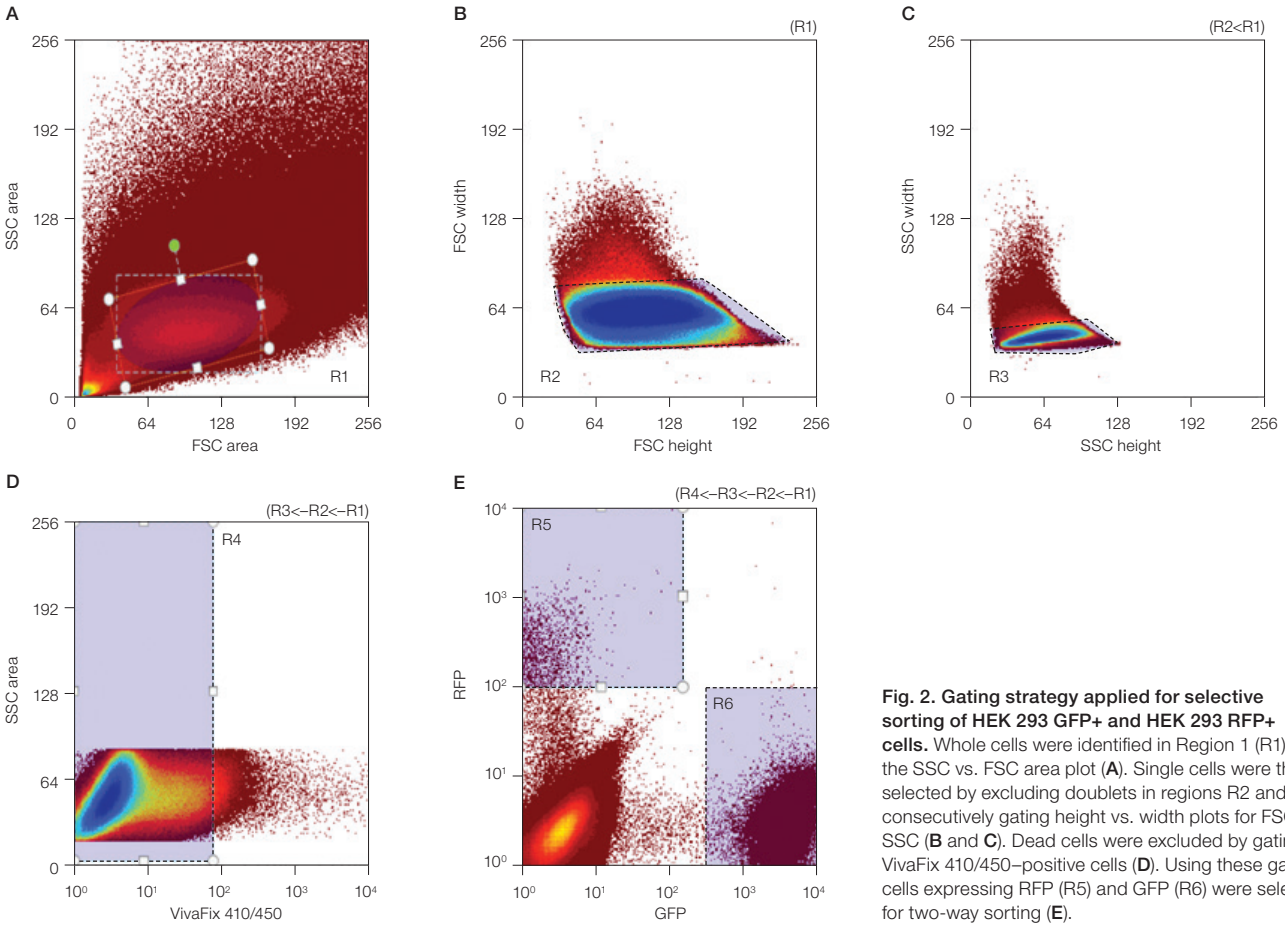
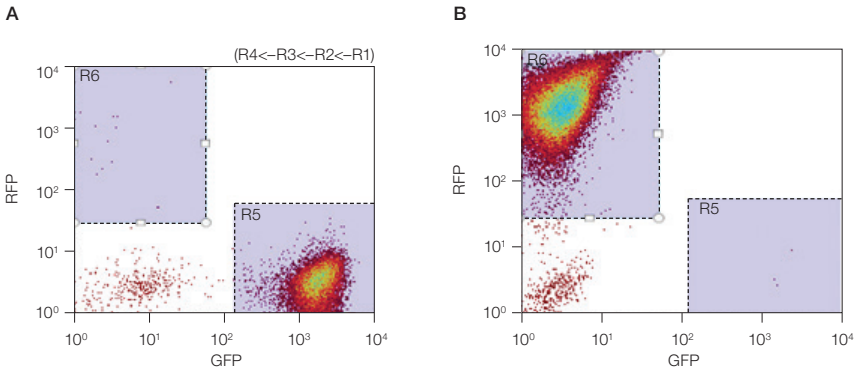


Fig. 2. Gating strategy applied for selective sorting of HEK 293 GFP+ and HEK 293 RFP+ cells. Whole cells were identified in Region 1 (R1) of the SSC vs. FSC area plot (**A**). Single cells were then selected by excluding doublets in regions R2 and R3 by consecutively gating height vs. width plots for FSC and SSC (**B** and **C**). Dead cells were excluded by gating out VivaFix 410/450–positive cells (**D**). Using these gates, cells expressing RFP (R5) and GFP (R6) were selected for two-way sorting (**E**).



Label	Count	% of Total	% of Plot
Total	24,307	80.6	100
R5	23,707	78.63	97.5
R6	29	0.1	0.1

Label	Count	% of Total	% of Plot
Total	63,026	91.1	100
R5	3	0	0
R6	62,290	90.1	98.8

Fig. 3. Analysis of sorted cells. We applied the same gating strategy used in Figure 1 to discriminate healthy single cells in the sorted cell populations. **A**, purity of the sorted HEK 293 GFP+ population was 98%, with 80% recovery. **B**, purity for the sorted HEK 293 RFP+ population was 99%, with 92% recovery.

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Conclusions

Cell health and purity following cell sorting are critical needs for any research application, but are especially important when working with rare cells. The prolonged run times and lack of accuracy inherent in many cell sorters present unique challenges for researchers working with rare cells. In this study, we showed that the S3e Cell Sorter can rapidly sort adherent HEK 293 cells that stably express either GFP or RFP, in a two-way sort at high purity and over 99% post-sort viability. The two key technologies that make this possible on the S3e Sorter are jet-in-air sorting coupled with the ProDrop feature, which enable researchers to sort rare cells at very high speeds with high recovery and ultra-high purity and viability.

The newly released three-laser S3e Cell Sorter provides more flexibility across a variety of fluorescence-activated cell sorting–based applications throughout a wide range of disciplines. Here, we used the three-laser configuration of the S3e with 405 nm, 488 nm, and 561 nm wavelength lasers to achieve accurate sorting of GFP+ and RFP+ cells. Very little compensation was required with this configuration as it allowed us to use a viability dye excited by the 405 nm laser in combination with the easily separated GFP and RFP selection markers. Further, adding the neutral density filter in front of the FL2 detector brought the bright signal from the HEK GFP+ cells into scale with our negative control cells, resulting in a healthy population of sorted cells expressing our selection marker.

The S3e Cell Sorter offers crucial advantages for applications involving rare cells that remain unmatched in other systems. The system is amenable to cell types as diverse as yeast, bacteria, and plant cells (Bidlingmaier et al. 2016, De Clerck et al. 2018, Liu et al. 2016) and can be applied to cells carrying up to four fluorescence- or dye-based selection markers. This application illustrates the utility of the S3e Cell Sorter in performing high-purity sorts of intracellular fluorescent proteins expressed in adherent cells.

References

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De Clerck O et al. (2018). Insights into the evolution of multicellularity from the sea lettuce genome. Curr Biol 28, 2,921–2,933.

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