Practical Issues in High-Speed Cell Sorting

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ABSTRACT

Modern flow cytometric cell sorters are all capable of so-called "high-speed sorting." However, there is confusion about exactly how fast a "high-speed" cell sorter can sort cells. There are many considerations in achieving the fastest sorting speed, as well as the highest quality sort results—cell recovery, purity, and functionality. This requires the same considerations required for "slow-speed sorting"; however, a more precise implementation is required for high-speed sorting. The modern cell sorters enable high-speed sorting because of advances in high-speed electronics and data processing. We discuss the practical considerations of high-speed sorting in terms of the theory and practical aspects of the mechanical and software components of sorting, statistics of sorting, cell preparation and viability, instrument setup, sort strategies, and biosafety. *Curr. Protoc. Cytom.* 51:1.24.1-1.24.30. © 2010 by John Wiley & Sons, Inc.

Keywords: flow cytometry • sorting • FACS

INTRODUCTION

Flow cytometry analysis and sorting have transformed biomedical research since their introduction in the late 1970s. Initially, nearly all flow cytometers were sorters but that has changed where "analytical" instruments, not capable of cell separation, now predominate. Nevertheless, sorting is a major application of flow cytometry and no other cell separation and purification strategy can combine the speed, purity, recovery and, particularly, the multi-parametric capacity that this technology offers. In the early days of sorting, it was rare to be able to purify sufficient cells for many types of subsequent analyses, e.g., mRNA purification, cell culture, and cell transfer, unless the starting sample had a large frequency of the desired cell population. Either approaches had to be developed that could get by with small numbers of cells or large amounts of time were required. The latter, however, often sacrificed cell viability and health. Early sorters were slow and frequently cantankerous and needed highly skilled and dedicated operators. While a skilled operator is still a requirement, current instruments are in general much easier to operate and are more dependable. Advances in electronics coupled with advances in partner biomedical technologies have transformed the once slow sorter into an instrument that is now much more practical for obtaining sufficient number of sorted cells, especially those of rare frequency. Many of these advances have also contributed to instruments becoming more reliable and easier to operate. High-speed sorting was born with the development of instruments capable of sorting metaphase chromosomes as the starting point for the human genome project (Peters et al., 1985). The instrumentation matured with subsequent developments in mechanics and electronics (van den Engh and Stokdijk, 1989; Van Dilla et al., 1990). The transition to commercial high-speed sorters occurred with the introduction of the MoFlo by Cytomation, Inc. in 1996. Now all sorters commercially available are capable of sorting in ranges generally considered to be high speed. However, while the instruments may process cells at high speeds, the resultant sorted cells—both in terms of quality, viability, purity, and quantity—depend on an understanding of core flow cytometry sorting principles and statistics. In this unit, we will try to demystify high-speed sorting and provide some solid general principles and practical suggestions for successful high-speed sorting.

WHAT IS HIGH-SPEED SORTING?

In this unit, we are limiting our discussion to the so-called electrostatic droplet sorters. Other sorters that use mechanical separation and laser ablation will not be discussed as these represent a very small subset of the sorters in use and the former have limited application

Current Protocols in Cytometry 1.24.1-1.24.30, January 2010
Published online January 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471142956.cy0124s51
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due to their slow speed. In the simplest sense, high-speed sorting means putting cells past the laser interrogation point at "high speeds," i.e., many cells per unit time. However, this definition can mean different things to different investigators and can often lead to unrealistic expectations from the instrument. High-speed sorting, of course, is meaningful only in the context of what "slow speed" sorting was. Thus, high-speed sorting means going faster than we could go before but does not necessarily mean a certain speed in an abstract sense. As we will see, how fast one can go with "high-speed" sorting is dependent on many factors and the resultant speed may seem "slow" to some but is always faster than could be done with the older "slow-speed" generation of instruments.

While most would expect that high-speed sorting means putting a high number of cells per second past the detector array, it also means how quickly the investigator can receive the sorted cells. While dependent on the cells per second consideration, this is also dependent on being able to set up the instrument quickly, have the instrument operate easily and reliably, and provide accurate readout of the instrument setup and sort progression. These are requirements because high-speed sorting necessitates that the instrument operate at a very high efficiency in all aspects to obtain the highest throughput.

High-speed sorters are also required to generate a high frequency of droplets into which the higher frequency of cells presented to the sorting system may be partitioned for sorting. The frequency of droplet generation is a function of the nozzle tip diameter, the sheath pressure, and the fluidic viscosity. Commercial high-speed sorters are expected to be able to generate up to 100,000 drops per sec (70-μm orifice), which enables cell input rates up to ~70,000/sec. However, as will be discussed, the sheath pressure, nozzle size, and input rate may vary substantially depending on the characteristics of the cells to be sorted and the desired result.

Regardless of the definition of "high-speed" sorting, physical characteristics of the mechanical and electronic components of the instrument and the nature of the particles/cells to be sorted sets an upper limit on the number of particles/cells that can be processed per second. To go faster requires additional approaches. One is some type of preprocessing of the cells to increase the frequency of the desired subset and decrease the total number of cells to process. Another is to use parallel processing, i.e., to split the sample between one or more identical sorting units that simultaneously process separate aliquots of the sample.

UNDERSTANDING HIGH-SPEED SORTING

Effects of Data Acquisition and Analysis on High-Speed Sorting

The successful use of modern sorters depends on understanding basic and advanced sorting principles. Especially dependent on this understanding is the ability to properly select the correct instrument components to match the type of cells being sorted, e.g., nozzle size and sheath pressure, and to match the instrument operation to the investigator's expectations of the sort result (if not unreasonable). The proper use of the instrument is paramount to obtaining high-quality results. Not doing so may adversely affect the purity and viability of the sorted cells.

Often overlooked is that the first step in sorting is effective cell analysis. It is as important for this front-end component of sorting as it is for cell analysis without sorting. The analytical part of the experiment should be optimized to obtain the best sorting result. In fact, it is best to optimize the analysis component on the instrument that will do the sort. While preliminary workup of a cell preparation and labeling can be performed on analytical instruments, it is advisable to verify that this procedure performs well on the sorter, especially sorters where the laser interacts with the cells in a fluid jet. One must verify that the cells of interest can be identified and resolved from other cell populations sufficiently to purify the population of interest without losing cells.

To sort fast, a sorter must be able to acquire data and process it very quickly. A sorter cannot sort faster than it can analyze and it cannot resolve cells into droplets and sort them if it cannot resolve cells during the analysis phase. If the instrument takes too long to process events, not all events will be processed and sort yield and, likely, purity will suffer. High-quality sorting can only be performed with high-quality data. The challenges for sorter electronics designers are demanding. Flow cytometer acquisition electronics must deal with noise that can be considerable and data that can have a large dynamic range. For high-speed sorting, data pulses can be very narrow (a few hundred nanoseconds) and data rates can be very high (tens of thousands per sec). In addition, analysis components such as

compensation place additional demands on the electronics.

The analysis yields of the acquisition software must match or exceed the ability of the sorter to isolate a high frequency of cells into sorted droplets (i.e., the sorting yield). If the acquisition system is not able to "see" a cell, it will not be possible to sort it. If the acquisition system cannot resolve cells adequately but can be informed that they are there (e.g., by doublet discrimination), large number of cells will be lost as these can not be sorted reliably. If the acquisition system cannot resolve populations adequately, the sort purity will also be sacrificed. Various electronics architectures have been developed for sorters but a detailed discussion of these is beyond the scope of this unit. Most of the commercial sorters currently being produced use "digital" electronics platforms. A fully digital flow cytometer does not exist. All cytometers, including sorters, are hybrid analog–digital systems. The signals produced by the PMTs/diodes are analog information, which is subsequently converted to digital data. In these "digital" strategies there are no logarithmic amplifiers, but rather the analog electrical data (current/amps) generated by the instrument photomultiplier tubes (PMTs) is converted to a voltage by the linear pre-amplifier. This analog voltage data is converted to digital information by high resolution ADCs. The ADCs are high speed—generally in the range of 10 to 100 MHz (10 to 100 million samplings per sec). The speed of the ADCs allows them to continuously sample the data (voltage) stream and permits essentially zero dead time acquisition. The high resolution of the ADCs produces data with a bit resolution (e.g., 16 bit ADCs convert voltages to digital values from 1 to 65,536) sufficient to produce logarithmic conversion. All sorters currently produced use at least 14-bit ADCs. Some instruments use a single ADC per channel, while others use two 16-bit ADCs per channel to effectively produce 23-bit data after combining the data from the two ADCs.

The ability of the acquisition system to resolve particles that are spatially close together, as is expected to occur frequently during high-speed sorting, where there is a high cell frequency, is critical for high analysis yield. This resolution ability is dependent on the pulse width, which varies with velocity of the cell past the laser beam, the size of the cells, and the height (dimension in the direction of the cell flow) of the laser beam. The electronics architecture (processing speed, baseline restoration, etc.) also affects the data resolution ability of the instrument. Older slow sorters used fixed-window pulse analysis, which had a considerable "dead time" that resulted in cells being missed if they arrived during a previous cell's window. The use of high-speed ADCs has eliminated this limitation. However, the gain may not be as much as many think. Many of the cells that were in the same analysis window in the older style of electronics still are too close to be resolved at the sorting stage of processing. Nevertheless, the development of electronics that permit the high resolution of cells is a major contributor to our ability to sort faster. Of course, proper operation of the sorter also affects the instruments ability to resolve cells. We will discuss this below as a discussion of core stream diameter.

Effects of Cell Concentration and Sample Core Stream Diameter

It should be obvious that to sort very fast we must be able to present cells to the system at a high rate. To do so requires that the cells be at a sufficient concentration to provide a high flow rate but at a sample volume injection rate that permits the maintenance of a small, relative to cell size, core stream diameter. The fluidic stream in any flow cytometer consists of a central stream containing the cells (called the core stream or sample stream) and a surrounding stream of sheath fluid. Single-cell resolution and measurement (the principle power of flow cytometry) will occur best when cells are in a single file relative to the laser beam and restricted to a reproducible path through the energy profile of the laser beam. The diameter of the core stream is dependent on the differential pressure between the sample and the sheath fluids. As the sample pressure is increased to raise the cell analysis rate, the ability to resolve single cells will decrease and electronic yield will also decrease reducing, of course, the effective sort collection rate. For best sorting, doublet discrimination strategies should always be employed to reduce the frequency of (it can never completely eliminate) doublets, which might be sorted under certain circumstances.

Factors Affecting Fluorescence Sensitivity and Population Resolution

The ability to resolve populations of cells with different levels of fluorescence is critical to the ability to sort these populations from each other. Populations whose fluorescence differs by large amounts are easy to resolve, but when the differences are small, it is more difficult. The ability to resolve real differences

in fluorescence is dependent on several factors. The ability to maximally excite the fluorochromes is dependent on the number of photons available to the cell from the excitation source—typically a laser beam—and this is dependent on the laser beam intensity and the dwell time of the cell in the laser beam (as well, of course, as proper alignment of the optical system). In all flow cytometers the cell is moving through the stationary laser beam and so the excitation source must have a very high light flux—i.e., provide many photons per unit time. Laser beams are best at providing the light flux needed, as the beam is small and coherent. In all sorters, the cell is moving much faster than in a typical analyzer. Thus, higher laser powers are generally required to illuminate the cell with the same number of photons. For example, in a typical jet-in-air sorter with a sheath pressure at 60 psi, the cell velocity is ~28 m/sec. For a lymphocyte, the cell is in the laser beam for ~900 nsec. In cuvette sorters, cells typically travel slower while in the cuvette (about 6 m/sec at 60 psi with a 70-μm sort orifice), and so the cell dwells longer in the laser beam and interacts with more photons and produces greater fluorescence. The use of higher numerical aperture lenses (typically NA 1.2) for fluorescence collection in cuvette-based sorters provides an additional increase in signal.

The ability to collect the photons emitted from the fluorochromes also affects the sensitivity and resolving power of the cytometer. A well-designed optical system with optimized light collection optics, sensitive PMTs, and efficient optical filtration are required for maximum light collection. Sorters in which the laser intersects with the cells in the fluid stream have an additional issue. The fluid stream acts as a lens causing the laser light to be spread into a narrow disk of light. This high intensity light must be excluded from the light collection path or it would obscure the fluorescence collection in paths at/near the same wavelengths (like trying to see the stars during the daytime). This is typically done with an obscuration bar to physically block most of the light. The obscuration bar, however, does not block all the laser light and does block some of the fluorescence light that we would prefer to collect. In cuvette sorter systems, and in all analytical flow cytometers, this issue does not arise as the laser interaction with the cells in the cuvette does not lead to the laser light disk and, thus, an obscuration bar is not necessary. Thus, cuvette sorters can be more sensitive than jet-in-air sorters (particularly notable at certain wavelengths, e.g., long red).

The purity of the sorted cells is affected in the analysis phase by the ability to resolve the populations and to properly position regions on which sorting is to be based (see *UNIT 1.8* for a general discussion of gating and *UNITS 1.20 & 1.21* for information on fluorescence sensitivity and resolution of dimly fluorescent populations). Sorting of populations with small fluorescence differences can present challenges. When the two populations, which are close together, are both brightly staining the populations, they are more easily resolved and sorted. However, if the two populations are very dim (e.g., very dim and negative), it is very difficult to resolve these cleanly over the course of the sort. It must be recognized that the measurement of the fluorescence from a single cell can have a very large variance. This variance accounts for a larger proportion of the signal from dim particles than from very bright particles—negative cells proportionately have the largest variance. As a result, population distributions of cells with low or no fluorescence have wider distributions, i.e., larger standard deviations. In addition, since we frequently visualize this on a logarithmic plot, the distances between dim populations can appear the same as between populations that are at much higher fluorescence intensities. For example, in the first decade of a log display, the values are very small and relatively large graphical distances represent very small actual differences with large errors. It must also be recognized that fluorescence measurements are not absolute measurements. If we assume that we make multiple measurements of a single cell (or single measurements of many theoretically identical fluorescent particles/cells), we would find that the measurements distribute around a mean/median. This is due to the fact that the fluorescence measurement process consists of a set of probabilities at several points. Each time an identical cell is analyzed we will find that there is a probability of how many of the fluorochromes are excited (or how many times), how many of the photons emitted from the fluorochrome are collected, and how many photoelectrons are produced as the photon shower interacts with the photocathode of the PMT. In addition, voltages can be generated by nonfluorescent events, often referred to as "noise or background" resulting from the instrument electronics or extraneous sources of light. These signals generally are of low intensity and would distribute within the first two decades and contribute to the

broadness of the negative population, making the resolution of dimly fluorescent cells from negative cells more difficult as they may overlap. It is important that an operator evaluate a sorter's performance in resolving dim/negative populations regularly and frequently.

A convenient sample to use for testing resolution at various fluorescence levels is a mixture of beads of various staining levels. It is even more convenient when the beads in the mixture are stained with multiple fluorophores so the beads can be excited and emit fluorescence over a wide range of wavelengths. Mixtures of such beads are commercially available from several sources and can be calibrated to relevant intensity standards or stained cells (see *UNIT 1.3* for procedures to cross calibrate particle standards). It is important to note that the beads will almost certainly not have the fluorescence characteristics of the cells stained with particular fluorochromes. The relative fluorescence intensity of the beads compared to stained cells will vary from one instrument to another depending on the excitation intensity and wavelength and the filters used for detection. Therefore, comparison of the beads to relevant cell samples or fluorochrome-specific bead standards is essential for useful monitoring of performance.

Dimly fluorescent cells will have large measurement variance and intensely fluorescent cells will have smaller measurement variance as a function of the real intensity. The separation index (*UNIT 1.21*) is a quantitative measure of how well separated the positive population is from a negative population. When large numbers of negatives and small numbers of dim positives are to be sorted, the negatives with high variance can be in the sort region and will be sorted. When the sorted cells are reanalyzed, the high standard deviation negatives will redistribute into the same distribution the negatives had prior to sorting and the sort will appear to have been ineffective with a low purity. Thus, for separation of dim populations, it may be necessary to bias the sort regions to the higher intensity side of the population, which will sacrifice recovery of some of the dim population cells but will favor exclusion of the dimmer/negative population from the sorted cells, providing higher purity (see Fig. 1.24.1). As with all sorting decisions, purity versus yield/recovery must be weighed.

When performing sorts using more than one fluorochrome, and where the emission from one or more fluorochromes contaminates the detection channel for another fluorochrome, one must use compensation to correct the spillover. Proper compensation and proper selection of fluorochrome panels will be critical for the proper separation of populations and placement of sort regions. We will not attempt a thorough discussion of compensation or fluorochrome selection here but refer the reader to *UNIT 1.14* and Maecker et al. (2004), respectively.

The decision as to where negative populations and dim positive populations separate can be difficult, due to the spreading of the data following compensation. This effect is compounded as the number of fluorochromes being compensated increases, since spillover of each fluorochrome contributes to increased spread in other and potentially multiple parameters (see *UNIT 1.21* and particularly the discussion of Fig. 1.24.5 regarding spillover). As a result, it can often be difficult to determine where positive and negative delineations should be drawn. The merits of isotype controls have been refuted as being useful in this regard. Alternatively, the use of Fluorescence Minus One (FMO) controls have been shown to be more effective in determining where to delineate populations. These controls contain each fluorescent marker minus one of the panel. In theory, there should be a FMO control for each antibody in the panel; however, in circumstances where populations are clearly separated and resolvable they may not be necessary. In general, when an epitope is expressed as a continuum from the negative population, or when the positives are only slightly brighter that the negatives, the use of a FMO control will be necessary to determine where to set positive/negative gates. For a more in depth discussion of compensation and FMO controls, please refer to *UNIT 1.14*.

The "Mechanical" Components of Sorting

Above we have discussed how issues with data acquisition and analysis can affect the outcome of high-speed sorting. The acquisition issues are, however, only the first step in effective high-speed sorting. Obviously, the electronic and mechanical components of sorting must also function properly. These electronic and mechanical components are, of course, responsible for generating the droplets, charging the droplets, determining and maintaining correct drop delay, and separating the charged droplets, allowing them to deposit into some collection vessel. Flow cytometric droplet sorters all work using the same physics, statistics, and other principles, and so all must follow the same general approaches

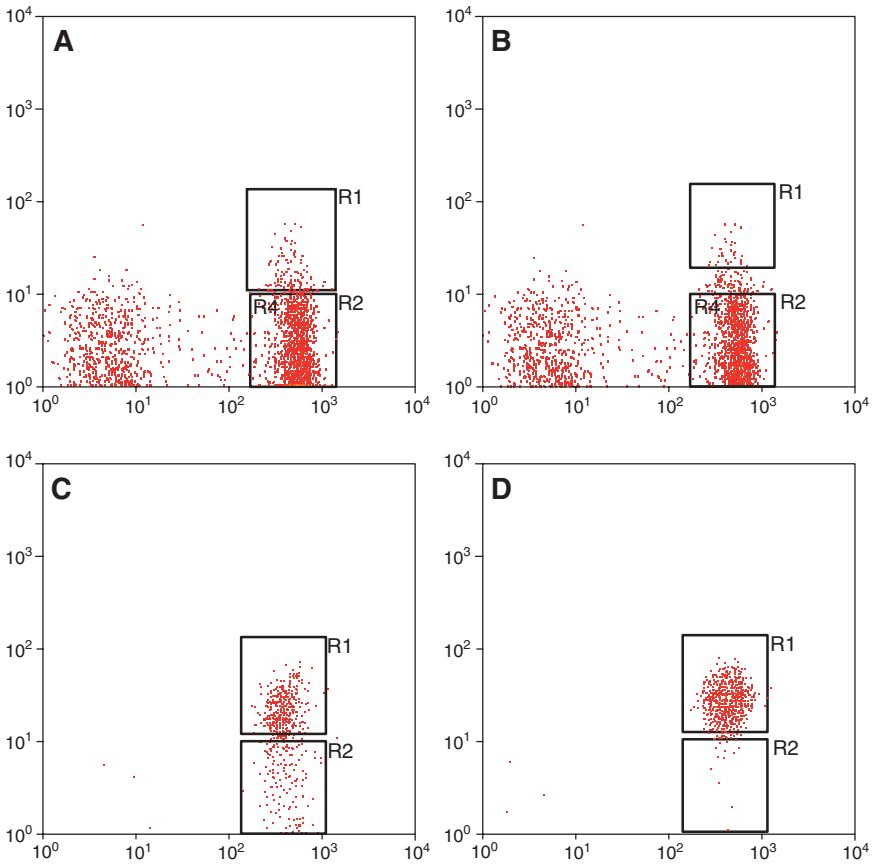

Figure 1.24.1 The histograms shown demonstrate the issue with sorting dim populations from negative populations. Panels **A** and **B** show the data from the starting sample. Panel A shows the position of R1 that tries to encompass the majority of what appears to be the positive sort region R1 population. Panel B shows a more conservative position for sort region R1 to emphasize purity. Panel **C** shows the sorting result using panel A R1 sort region and panel **D** shows the sort result using the panel B R1 sort region. In panel C, the sort result is heavily contaminated with negatives while in panel D the sort result is much more pure for the positive population. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cy0124*.

to the problem and, despite some sales claims that would require the contrary, cannot violate these principles.

Generating droplets

Droplet sorters work on the following basic description (see *UNIT 1.7*). A fluid jet (always in air regardless of where the cell analysis takes place) is broken precisely into discrete droplets by applying an acoustic energy to the fluid, which following Raleigh's formulae (Rayleigh, 1877) will break into droplets at some point from the origination of the stream. The cells in the fluid stream partition into these droplets and droplets containing cells of interest are charged electrically through the fluid stream as they break off. The droplets retain the charge and are thus deflected as they travel through an electrostatic field and are collected in one or more types of collection vessels.

The first step in the process is the stable generation of droplets. A thorough discussion of the physics of the generation of fluid jets and drops is beyond the scope of this unit (see Pinkel and Stovel, 1985 and van den Engh, 2000 for additional information). A fluid stream breaks into drops based on the force of surface tension. The fluid tends to form drops but the drops remain part of the fluid stream until they reach a size where surface tension can no longer hold the drop in the stream. At certain low pressures, the formation of the drops will be random and uneven. However, as the stream velocity increases due to increasing pressure behind the stream, the stream will break into more uniform droplets. A relationship exists between the diameter of the fluid stream and the velocity of the stream. Rayleigh described this relationship in 1877 (Rayleigh, 1877) and showed that a stream of

fluid will break into drops when the wavelength of the perturbations along the stream exceeds the stream diameter (D) times π ($\lambda = \pi D$). The optimal wavelength of the stream perturbations works out to ~4.5 D. The spacing and, thus, the drop rate are proportional to the stream velocity. Thus, to generate more drops/sec with a given stream diameter (i.e., nozzle tip orifice diameter), we must increase the stream velocity and do so by increasing the pressure behind the fluid stream.

In order to stabilize the droplet formation and to shorten the distance from the orifice that the droplets will form, an acoustic energy is applied to the fluid stream. This acoustic energy can be varied in both frequency and magnitude (amplitude). Acoustical energy is produced by having a piezoelectric crystal coupled to the fluid stream. The vibrational frequency and amplitude of a piezoelectric crystal changes with a change in the frequency and amplitude of an electric voltage placed across it. The crystal is not in actual contact with the sheath fluid but rather focuses the energy to the fluid through the nozzle body. A stream of given diameter and velocity (sheath fluid pressure) will have an optimal frequency and amplitude (or perhaps a couple of resonant energies) where the droplet formation is most stable and will yield droplets of proper size. Generally, the optimal drop frequency, at a given stream velocity, will be the one which produces close to the shortest length from the nozzle to the formation of drops (the breakoff point). This assumes that the general criteria for proper drop formation are met—i.e., the Raleigh conditions can be satisfied, the available drop drive energy requirements can be satisfied, and for sorters that analyze in the fluid stream that stream perturbation at the analysis point is minimal. In order to sort faster, we must increase the concentration of the cells in the fluid stream. To avoid having more than one cell contained in each droplet we must generate higher numbers of droplets (i.e., we must use sheath fluid pressures of increasing magnitude) to, on average, partition only single cells into each droplet. Most sorters today, using a 70-μm tip orifice diameter with a sheath pressure of 60 psi, can produce drop frequencies in the range of 90,000 to 100,000 drops/sec. To generate higher droplet frequencies requires increases in pressures that are difficult and expensive to produce, are unsafe, or would be expected to have harmful effects on cells, and/or a smaller nozzle orifice diameter. Note that cell size and/or shape may dictate larger nozzle orifice diameters that will require lower pressure and stream velocities, which in turn will require lower frequencies of droplets and, thus, lower cell numbers processed per sec—i.e., slower sorting.

Analysis, sorting, and charging coordination–drop delay

The analysis of the fluorescence properties of the cells and their inclusion or exclusion in a population to sort occurs, of course, when the cell is in the laser beam (or a few nanosec after). This point will be well upstream of where the fluid stream is breaking into droplets. A major challenge in sorting is to determine when the desired cell will be in the correct position—the last attached droplet—for charging the stream to allow the droplet to be sorted. This is referred to as the time delay (or drop delay) and must be measured and maintained very accurately over the course of the sort. If this does not happen, then sort purity and/or sort yield/recovery will be severely compromised. Under conditions of inaccurate drop delay, the system will charge the last attached drop (assuming charging phase is properly set) but the drop charged will not be the drop that actually contained the desired cell but rather will be the drop before or after. Not only will the desired cell not be sorted, but also the wrong incorrectly sorted drops will either contain no cell (reduced cell recovery) or will contain a cell that will simply be a random selection from the entire sample of cells. Post-sort analysis of such a sort will show relatively few cells and the distribution of the cells found will be the same as the starting sample. As we discuss below, sorter manufacturers have various approaches to determining the drop delay.

Not only must the stream charging be delayed properly, in terms of drop cycles to occur when the cell of interest is in the correct drop, but the drop formation oscillator (drop drive frequency or clock) and the sort charging systems clock must be in precise phase (i.e., synchronized) for proper timing of the charge application. The drop drive and the charging systems operate as a cyclical process and may be thought of as sine waves (as they properly are). The sine waves must overlay precisely for proper timing. The charging system must charge the stream just as the drop to be sorted is finalizing its separation from the stream. If it charges slightly early or late, the drop will not get the full charge intended. The synchronization is properly set when sorted streams are deflected the maximum amount (assuming the deflection field is held constant).

Drop formation stability

The major hurdle to overcome to provide excellent sorting is that the droplet formation remains very stable. This requires proper fluidics set up, and of course, proper electronics design (beyond the scope of this unit), such that droplet formation does not drift over time. Stable drop formation will be adversely affected by air bubbles in the sheath fluid, partial occlusion of the nozzle orifice, external vibrational energies, a change in the sheath fluid pressure, or the velocity of the sheath fluid caused by a change in sheath fluid temperature. Air bubbles in the sheath fluid and/or trapped in the nozzle will effectively change the efficiency of coupling of the acoustical energy from the piezoelectric crystal to the fluid. Efforts should be made to ensure that the sheath fluid (and sample fluid) do not contain or have otherwise introduced air bubbles into the nozzle. It is easy to recognize the introduction of air into the nozzle as the observed drop image will drastically shift. The data display, especially forward scatter, will also usually show a dramatic change. These changes—if not correctable by removal of the air bubble (preferable)—may be corrected by adjusting the crystal drive amplitude. However, this will not correct any data perturbations. A change in temperature of the sheath fluid will affect the viscosity of the fluid and, therefore, will affect the way the droplets form by changing the wavelength of the propagating energy in the fluid stream. This will alter the break-off timing and should be adjusted by also changing the crystal drive amplitude. It is necessary to reduce temperature fluctuations by having an adequate HVAC system in the sorter room and by moving heat-generating components away from the sheath tanks, fluidics lines, and nozzle. The sheath fluid tanks and the line that deliver the sheath fluid to the nozzle must be isolated from environmental vibration. Additional vibrational energy will affect the total vibrational energy, which will affect the drop break-off position (while monitoring the drop delay vibrate the sheath line and observe the effect). Changes in sheath fluid pressure will affect the drop break-off position but not the break-off timing. These changes will affect how far away from the tip the stream breaks off but not the timing. This is because these changes are affecting the stream velocity and, thus, the drop delay will be the same even though the breakoff distance is different. Use of the drop drive amplitude to correct this will be inappropriate and the operator will actually be changing the delay and adversely affecting the sort quality.

The stability of drop generation is monitored using images from cameras with the drop motion stopped with frequency-synchronized strobes. Initially during set up, the droplet separation is adjusted to have an image where the last drop is attached to the fluid stream by a thin neck (see Fig. 1.24.7A). It is often useful to set the camera strobe phase to zero (or some small value, e.g., 5) to be sure that you have the drop formation correct relative to the charge timing. The image selected for monitoring must be maintained over the length of the sort or adjusted as described above. Operator initiated adjustments can be made if the operator is willing to constantly monitor the image and make the necessary adjustments. If this is the preferred method, it is useful to mark reference lines on the image monitor. More recently, sorter manufacturers have included image analysis feedback loops to maintain the drop image by adjusting the drop drive amplitude. These monitor the system constantly and frequently—every second or so—and can detect changes and attempt to correct the image. If they are unable to maintain the image, they can stop the sort and alert the operator. A sensitive indicator that the sort conditions are varying is observed changes to the side streams. As the breakoff drifts, the synchronization of the drop formation and the stream charging separates and the drops do not get the full charge and, thus, they are deflected differently.

Sort stream integrity and control

The sorted streams (or so-called sidestreams) need to be maintained in proper position as discussed above. Sort stream fanning (spreading) can occur for a number of reasons. The first is a phenomenon that all sorters must deal with. When a drop is charged (assuming perfect charge phase), it will induce an opposite charge in adjacent drops. If that adjacent drop is also to be charged with the same polarity as the first drop, the induced opposite charge will partly offset the applied charge—i.e., the second drop will be charged slightly less than the first drop. When these two drops travel through an identical deflection field, the one with the greater charge will deflect more and travel on a slightly different trajectory than the original drop. Thus, the width of the sort stream will increase, or we say the side stream is fanning. If the second drop is to be charged with the opposite polarity from the

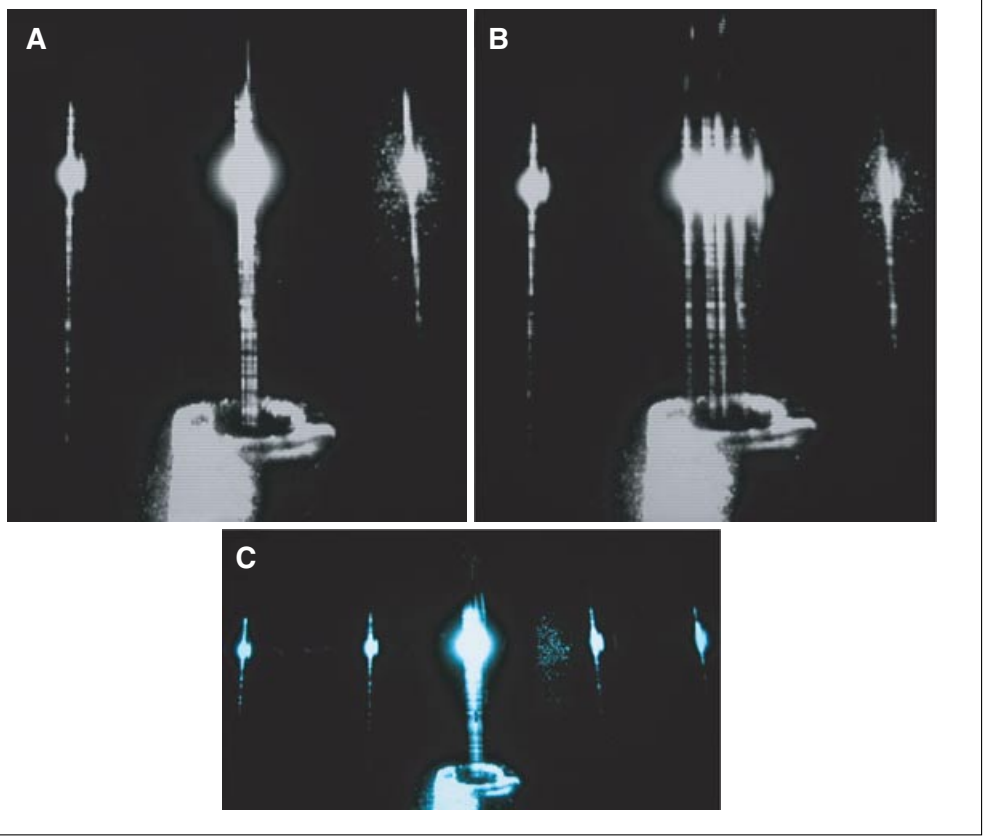

Figure 1.24.2 These photos (A and B) from the stream illumination camera show a central waste stream and two deflected side streams. Panel **A** shows the waste stream after proper defanning has been set. Panel **B** shows the waste stream before adjustment for charge correction (i.e., defanning). Panel **C** shows a photo of the setup for 4-way sorting.

first, the charge on that drop will be more (applied charge plus induced charge) than another drop charged fully with that polarity leading to the same problem but in the other sort stream. This is compounded with the ability of sorters to sort up to two populations in each polarity direction (total of four streams). The charge induction has to be taken into account and adjustments made to the adjacent drops. Thus, when an adjacent drop is to be charged with the same polarity, the charge amplitude is increased slightly to offset the induced slight opposite charge. When the adjacent drop is to be charged with the opposite polarity, the applied charge amplitude is slightly reduced. The system is told how much (in percentage) to adjust by an operator selection and sophisticated systems can vary the charge over a span of about four adjacent drops. The fanning will also occur on the waste (unsorted) stream. A variable adjustment is made while visualizing the waste stream fanning and is adjusted until the waste stream fanning is reduced as much as possible (see Fig. 1.24.2). How well the system is designed to handle this is best determined by visualizing very high-frequency sort streams. High-frequency sort streams mean that charge induction is occurring constantly and affecting many adjacent drops. If not handled properly, these high-frequency side streams can fan to the point where some fraction of the cells may be missing the collection tube or contaminating an adjacent tube.

Fanning is always the result of producing sorted droplets that carry various charge amplitudes assuming that there is no malfunction in the charging electronics (rare). However, this can occur for other reasons than that just described above. When the charging electronics clock is not synchronized with the drop formation, the charge may be spread across two drops (probably unequally) causing improper charging of the breakoff drop. If equal-sized droplets are not generated, the droplet charge will vary as a function of the surface area of the droplet (charge is only carried at the surface of the droplet). Unequal-sized droplets can form because of perturbations to the droplet formation process. When the cell size becomes a larger fraction of the droplet size/stream

diameter and these cells occur near the boundaries of drops that are breaking off, the drop formation is altered to produce larger and smaller droplets. This effect is seen not only with larger cells but also because of cell shapes. In addition, large amounts of debris in the sample promotes side-stream fanning for the same reason. If the size and/or shape of the cell are the issue, the solution is to use a larger nozzle tip orifice diameter. If debris is the issue, the debris should be reduced or removed.

Sort decision strategies

In modern sorters, the position of the cell within the drop can be predicted with fairly good accuracy. Different sorters can resolve the position to within 1/32nd to 1/1000 of a drop period. The position sensing is done when the cell occurs in the laser—i.e., the system trigger criteria are met. The time of the triggered event is referenced relative to the system clock (a sinusoidal wave)—the time within the start and end of a clock cycle providing the position. This position, assuming the system clock and the drop formation clocks are synchronized, predicts the position of the cell within the drop. Cells arrive at the laser at random times that are described by Poisson statistics (see below). Thus, the cells also arrive at the last attached droplet at random times, but the drop formation is not random but constant at a predictable frequency. This means that cells can occur anywhere relative to the drop boundaries. When a cell occurs near a droplet boundary, there is a finite probability that the system has not correctly predicted its arrival at the drop or that the cell will partition into the droplet before or after the charged drop depending on which side of the drop period it occurs. It is possible to instruct the sorter to sort two adjacent drops when the cell position is predicted to be at the edge of droplets. These are referred to as sort modes or sort masks and the total sorted event (drops) or inspected event (drops in the sort masks) is termed the sort envelope. The operator may select how the system should perform based on fractions of a drop period. Cells which are predicted to be in the specified fraction of a drop at the leading or trailing edges will trigger the system to sort the predicted drop and the drop before (for a cell in the leading cell fraction) or after (for a cell in the trailing drop fraction). This will function to increase the probability that the cell of interest actually ends up in the collection tube. However, this can cause a loss of sorted cells when the cell rate is high, as the likelihood that a sort envelope will be contaminated by an unwanted cell increases.

In old sorters where the electronics were not as good at being sure where the cell was, we often specified either a 2-drop or a 3-drop sort envelope. This would ensure that we captured the desired cell, but if the cell event rate increased this could lead to substantial loss of desired cells as sort envelopes of 2 and 3 drops have a higher probability of containing an unwanted cell. This in part contributed to the slower speeds of the older generations of sorters.

It is also important that the positions of other cells, especially unwanted cells, close to the desired cell be predicted. The system can then predict if these unwanted cells will contaminate the sort envelope and, if so, can determine, based on operator input, how to deal with the contaminating cells. The sort mask (also called exclusion zone or reject width) can be set in most instruments to a width specified as a fraction of a drop. The mask is operative on both sides of the predicted drop. The width of the sort mask that is desired is based on a choice between purity and yield. The sort/exclusion mask will of course cause a loss of a fraction of the desired cells since when an unwanted cell occurs within the sort mask area, the sort event will be aborted, causing the loss of the wanted and unwanted cell. Depending on the experimental needs, different types of sort masks can be set.

When higher purity is required, a purity mask is set. In this mode, the user specifies how far on each side of the predicted drop one wishes to look for contaminant cells. Figure 1.24.3 illustrates a purity/exclusion mask of 0.25 (i.e., one quarter) of a drop on either side, thus, the sort mask is 1.5. Any unwanted cells that occur within the 0.25-drop width on either side will trigger the system to abort the sort. Operators should understand the consequences of increasing the purity mask width—lower yield but higher purity.

If a higher yield is required, one can set a yield/recovery mask. In this mode, the sorter will sort either one drop or two drops. If the cell is predicted to be in the middle fraction of the drop (usually the middle half but on some instruments can be specified), the sorter sorts just the one drop containing the cell of interest. If the cell is predicted to be at the edge (either leading or trailing) of the target drop, the sorter will sort two drops. If the cell is at the leading edge, then the target drop and the drop before it will be sorted. If the cell is predicted to be in the trailing edge of the drop,

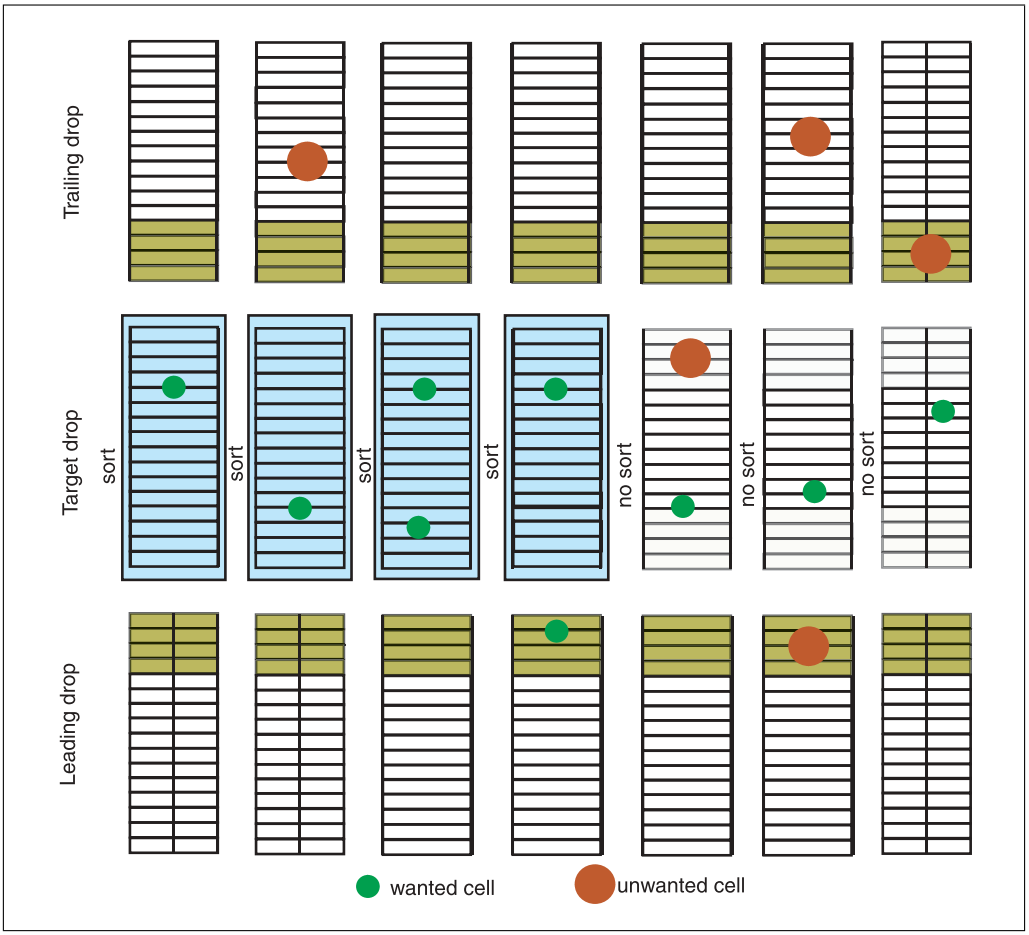

Figure 1.24.3 This illustration demonstrates the effects of setting a purity mode (or mask). The rectangles represent the drops to be formed. Note that at the time these sort decisions are being made, the "drops" are not yet formed but are contained in the fluid stream. The "target drop" will be the drop which is predicted to contain the desired cell to be sorted, the "leading drop" will be the drop to be formed immediately preceding the target drop, and the "trailing drop" will be the drop to be formed following the target drop. These definitions apply to all similar figures. The drops are divided into 1/16ths (for simplicity—current instruments have divisions which range from 1/32nd to 1/1000th) for cell localization. The exclusion zone (or purity mask) that has been set is 0.5 drops (i.e., 0.25 drops to either side of the drop predicted to contain the cell to be sorted. This means that the sort envelope is 1.5 drops). The small (green) dots represent wanted cells and the large (red) dots represent cells that are not wanted. The drops that will be sorted are boxed (light blue). For the color version of this figure go to *http://www.currentprotocols.com/protocol/cy0124*.

then the target drop and the trailing drop are sorted (see Fig. 1.24.4). Yield masks can be combined with purity masks (see Fig. 1.24.5).

If one wishes to emphasize recovery of all wanted cells but can tolerate lower purities, then a yield mask of perhaps 1.5 to 2.0 (0.25 or 0.5 drops on either side) should be used. At the extreme of this mode is the enrich mode. In this mode, the sort aborting system is turned off altogether and the system pays no attention to contaminant cells but sorts solely to recover all wanted cells without regard to any contamination. This mode is frequently used when one has a very low frequency of desired cells and wishes to concentrate them. The sort can be run at very high trigger rates—up to ~75,000 cells/sec. This will provide an enriching sort at maximum speed but which is likely to be far less pure than desired. Subsequent to the enrich sort, a purify mode sort can be performed on the product of the enrich sort to increase the purity. This can usually reduce the total sorting time.

The final sort mode or mask that can be performed is the single cell (or phase) mask. In this mode, the only time a cell will be sorted is when the cell occurs in the middle of the target drop (see Fig. 1.24.6). If the desired cell is in some outside fraction of the drop (e.g., 0.25), the sort is not performed. In addition, the occurrence of any other cell—even a wanted cell—within the sort envelope will abort the

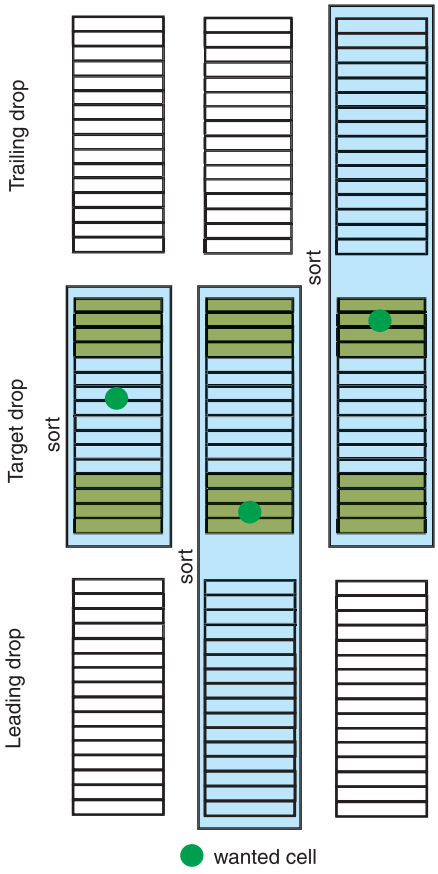

Figure 1.24.4 This illustration shows the effects of setting a recovery or yield mask of 0.5 drops (0.25 drops on either side of predicted drop). When the cell is in the middle half of the drop predicted to contain it, only the one drop will be sorted. When the cell is in either of the outside quarters of the drop, the drop predicted to contain it and the drop to the respective side will be sorted as the cell may partition into the neighboring drop. The drops that will be sorted are boxed (light blue). For the color version of this figure go to *http://www.currentprotocols.com/protocol/cy0124*.

sort. This mode can also be combined with a purity mask. Single cell modes are used when one wishes to know an exact count of how many cells are deposited. This mode should be used when cloning cells or placing other defined numbers of cells into tissue culture plate wells or PCR tubes. The single mode results in the highest loss of wanted cells, but usually this is not a problem, as relatively few sorted cells are needed. If the frequency of the wanted cells is very low and a relatively large number of cells are needed and/or if the sample size is limiting, one may have to rethink the sort mode. In this case, a single drop purity sort (with or without an exclusion zone) might be considered, recognizing of course that the probability of any given well receiving a cell is lower and that any given well might receive two wanted cells (unlikely at very low wanted cell frequencies).

Sorting statistics

An understanding of the statistics affecting sorting is critical to understanding how to set sort modes and other factors that affect the expected yield and purity. The Poisson statistic describes the probabilities of the occurrence of random events. Since we want to sort and make meaningful decisions about what cells we get, as we discussed above, we need to understand the statistics involved so we can decide how many cells we can push through the system; what purity and yield we might expect, and how long it will take to do the sort to get the number of cells desired. What we try to predict is how frequently we can expect coincident cells in the laser beam and at the break-off droplet. Three classes of coincidence have existed traditionally: (1) coincident cells are too close together to be resolved by the analysis component of the sorter; (2) coincident cells

Figure 1.24.5 Shown in this figure is the effect of combining both a purity and recovery/yield mode or mask. Both masks are set to 0.5 drop (i.e., ± 0.25 drop). The yield mask is shown in olive (shaded) and the purity mask in light purple (hashed). Wanted cells are represented by small (green) dots and unwanted cells by large (red) dots. The drops that will be sorted are boxed (light blue). For the color version of this figure go to *http://www.currentprotocols.com/protocol/cy0124*.

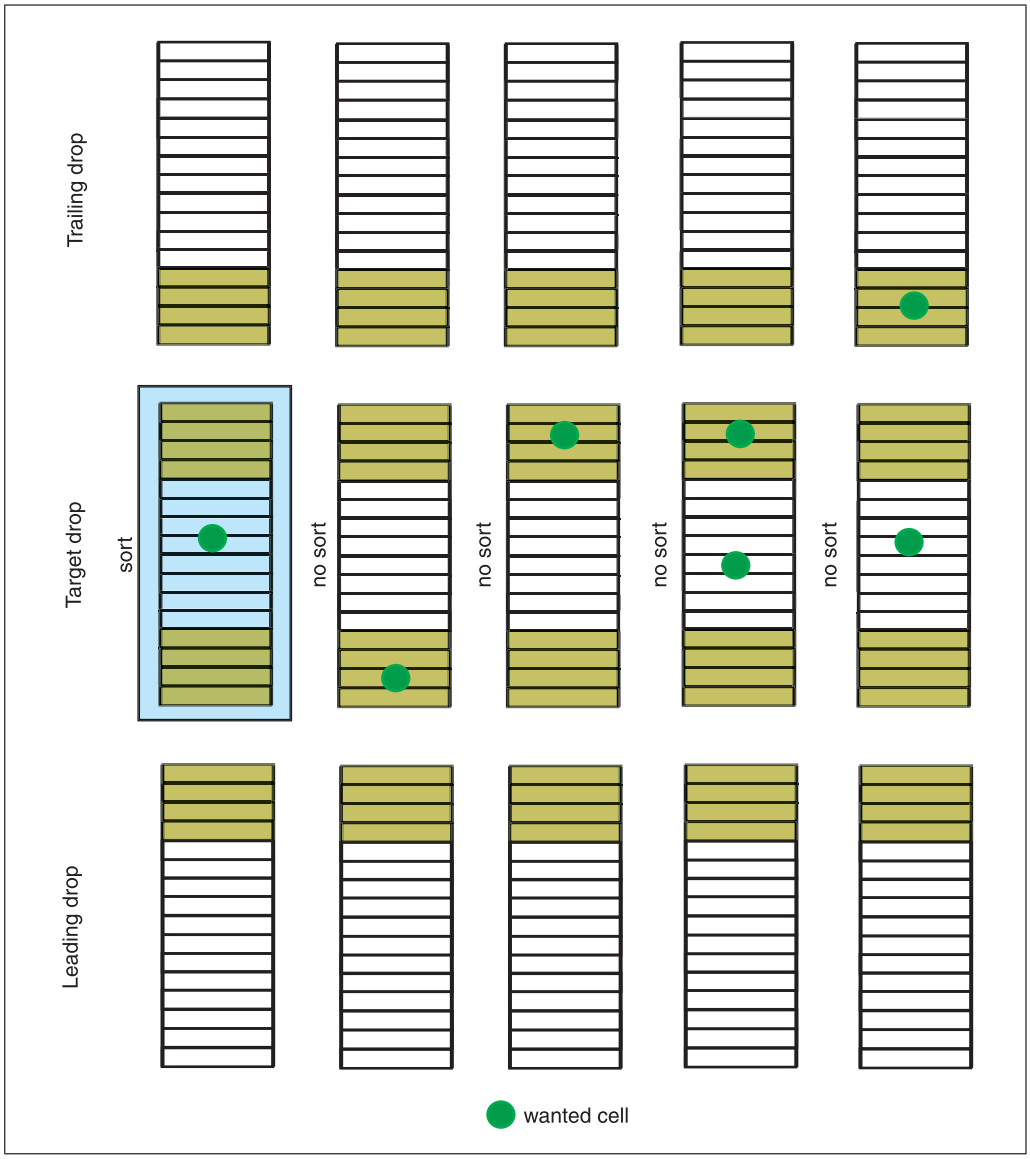

Figure 1.24.6 This is an illustration of a "single" sort mode (phase mask). The mask of 0.5 drops is shown in shaded (olive). The drops that will be sorted are boxed (light blue). Only when a cell is predicted to be in the middle half of the drop will the drop be sorted. This mode results in the largest loss of cells of any of the modes. Note the single mode can also be combined with a purity sort (not shown here). For the color version of this figure go to *http://www.currentprotocols.com/protocol/cy0124*.

are identified as present by the analysis component but too close together to resolve—in the modern (so-called digital) instruments this is decreased as faster electronics allow individual analysis windows for each event (note an event can still be 2 cells); (3) coincident particles are detected/resolved but are too close together to be separated at the sorting stage. Thus, most coincidence in sorting in modern sorters (when cells are resolved by the analysis system as individual events/cells) comes from the third class of coincidence. Note that the ability to perform doublet discrimination (using pulse processing analysis) to identify class 1 coincidence event does not change this as it will usually (but not always) eliminate these coincident events. Note also that the ability to resolve doublets and other coincidence events (class 1) is dependent on the relationship between the size of the particle and the laser beam height. Thus, our discussion applies to those cell types (the most common applications) for which the cell size is close to, or larger than, the laser beam height (e.g., lymphocytes and larger cells). The less common exceptions are for those small particles (e.g., bacteria, yeast, or platelets) where the laser beam height is larger than the particle and,

thus, where two discrete cells are fully within the beam simultaneously. These events would not fall into the class 2 or 3 coincidence groups, but would behave like a class 1 coincidence event.

The third class of coincidence, and, thus, the sort rate, can be calculated from the Poisson statistic (see Pinkel and Stovel, 1985). The formula for the calculation of the best sort rate is: $Rs = \varepsilon\mu e^{-(1-\varepsilon)\mu Tn}$, where Rs is the sort rate, ε is the fraction of cells to be sorted (i.e., wanted), μ is the input cell frequency (no. cells/μsec), T is the droplet period (1 sec/drop frequency per sec), and n is the number of droplets deflected per sort event or n is the number of drops examined in an exclusion sort mask. Note that n must take into account the entire sort mask. Thus, when using a recovery or yield mask and a 0.25 drop exclusion is set, the number of drops is 1.5—the drop to be sorted plus the exclusion of 0.5 drops. We have prepared an Excel spreadsheet for calculating sort rates that you may download and use (*http://flowcytometry.med.unc.edu/sorting.htm*). This calculation provides a better estimate of coincidence than does a simple application of the Poisson statistic. It can slightly underestimate the sort rate as it does not take into consideration the small frequency (in most sorts) of coincident cells both being wanted cells. By application of the binomial distribution ($[(p + q)^n = 1$), where p is the fraction wanted, q is the fraction unwanted, and n is the number of coincident events we wish to calculate, we can calculate this. However, this underestimation is offset by the fact that the "perfect" coincidence estimated is usually an overestimation of what real life coincidences will be when non-randomness is considered. Additional discussion of the statistics of cell sorting can be found in *UNIT 1.7*.

Real cells usually have some degree of clumping. In addition, and probably more importantly in modern sorters where we have good tools for revealing clumps, other factors can lead to cells not being independent and, thus, not subject to just random occurrence. Non-independence (i.e., cells are revealed as individual cells but are still not independent) can increase coincidence aborting dramatically. Below we present some example data of sort rate calculations using different sort conditions (tip size and drop frequency) and total and wanted cell rates. See Tables 1.24.1 and 1.24.2.

PRACTICAL ASPECTS OF HIGH-SPEED SORTING

Cell Preparation and Cell Viability

It goes without saying that good high-speed sorting is dependent upon good sample preparation. Samples containing cells with high viability, low clumping, and little debris are desirable. The nature of the starting cellular material will often dictate the preparation procedure. Certain tissue or cell types may be more problematic than others (e.g., lymphocytes are very easy while tissues e.g., liver and adult neuronal tissue are problematic). Embryonic tissues are usually much easier to disaggregate than are the same tissue from adults. Many factors can influence the viability of cell preparations. For adherent cells grown on tissue culture plates, always avoid mechanical scraping to remove the cells. This almost always results in a very poor preparation of cells with many dead cells, much debris, and clumping. Adherent cells should be removed by enzymatic and/or cation chelation (EDTA) and/or cold. It must be recognized that enzymatic removal may result in loss of surface protein and glycoprotein antigens. Difficult to remove cells may be successfully grown on and removed from low-adherence tissue culture plates. The use of polystyrene test tubes should be avoided in favor of polypropylene tubes. Conical-bottom test tubes should be avoided in favor of round-bottom tubes. Centrifugation steps should be of a duration that is only long enough to minimally pellet the cells and the cells should not remain in the pelleted state for any longer than necessary. It is best to be at the centrifuge when the rotation stops and immediately remove the tubes. Removal of supernatant from the cell pellet should be performed by pouring off the fluid rather than by suction removal, to avoid loss of cells. Immediately after removing the supernatant, the pellet should be disaggregated **before** any additional fluid is added. Clumps should be removed by filtration through a mesh filter (30-μm mesh; e.g., self-cut Nitex *http://www.industrialnetting.com*, or commercial filter units—Partec, *http://www.partec.com*; BD Biosciences, *http://www.bdbiosciences.com*; Miltenyi Biotec, *http://www.miltenyibiotec.com*) at each step, otherwise any clumps tend to act as a source for further clumping. It may be advantageous to include 200 μg/ml of DNase in the media through all steps to degrade high-molecular-weight DNA, which promotes clumping. The addition of up to 5 mM EDTA to the buffer will

Table 1.24.1 Example Sort Efficiencies for a 70-μm Tip at a Sheath Pressure of 60 psi[a]

Input rate (cells/sec)	% wanted cells	Input rate of wanted cells(/sec)	Best sort rate (cells/sec)	Wanted cells collected per hour	% sorted	Sort efficiency (%)
A. 70 μm tip – 60 psi – 93,000 drops/sec – 1 drop sort envelope						
5000	20.0	1000	958	3.3×10^6	19.16	95.79
10,000		2000	1835	6.6×10^6	18.35	91.76
15,000		3000	2636	9.5×10^6	17.58	87.89
20,000		4000	3367	1.2×10^7	16.83	84.19
25,000		5000	4032	1.4×10^7	16.13	80.65
30,000		6000	4635	1.6×10^7	15.45	77.25
5000	5.0	250	237	8.5×10^5	4.75	95.02
10,000		500	451	1.6×10^6	4.51	90.29
15,000		750	643	2.3×10^6	4.28	85.79
20,000		1000	815	2.9×10^6	4.08	81.52
25,000		1250	968	3.4×10^6	3.39	77.46
5000	1.0	50	47	1.7×10^5	0.95	94.82
10,000		100	89	3.2×10^5	0.90	89.90
15,000		150	127	4.6×10^5	0.85	85.24
20,000		200	162	5.8×10^5	0.81	80.82
25,000		250	191	6.9×10^5	0.77	76.63
5000	0.2	10	9	3.4×10^4	0.19	94.77
10,000		20	18	6.5×10^4	0.18	89.82
15,000		30	25	9.2×10^4	0.17	85.13
20,000		40	32	1.1×10^5	0.16	80.68
25,000		50	38	1.3×10^5	0.15	76.47
B. 70 μm tip – 60 psi – 93,000 drops/sec – 1.5 drop sort envelope						
5000	20.0	1000	937	3.3×10^6	18.75	93.75
10,000		2000	1,757	6.3×10^6	17.58	87.89
15,000		3000	2,472	8.8×10^6	16.48	82.40
20,000		4000	3,090	1.1×10^7	15.45	77.25
25,000		5000	3,621	1.3×10^7	14.48	72.43
30,000		6000	4,074	1.4×10^7	13.58	67.90

continued

Table 1.24.1 Example Sort Efficiencies for a 70-μm Tip at a Sheath Pressure of 60 psi[a], *continued*

Input rate (cells/sec)	% wanted cells	Input rate of wanted cells(/sec)	Best sort rate (cells/sec)	Wanted cells collected per hour	% sorted	Sort efficiency (%)
B. 70 μm tip – 60 psi – 93,000 drops/sec – 1.5 drop sort envelope, *continued*						
5000	5.0	250	232	8.3×10^5	4.63	92.62
10,000		500	429	1.5×10^6	4.29	85.79
15,000		750	595	2.1×10^6	3.97	79.47
20,000		1000	736	2.6×10^6	3.68	73.60
25,000		1250	852	3.0×10^6	3.41	68.18
5000	1.0	50	46	1.7×10^5	0.92	92.33
10,000		100	85	3.1×10^5	0.85	85.24
15,000		150	118	4.2×10^5	0.79	78.70
20,000		200	143	5.2×10^5	0.73	72.66
25,000		250	167	6.0×10^5	0.67	67.09
5000	0.2	10	9	3.3×10^4	0.18	92.27
10,000		20	17	6.1×10^4	0.17	85.13
15,000		30	23	8.5×10^4	0.16	78.55
20,000		40	29	1.0×10^5	0.15	72.47
25,000		50	33	1.2×10^5	0.13	66.87

[a]Sort efficiencies are calculated for a 70-μm tip at a 60 psi sheath pressure at various input rates and wanted cell frequencies. Table 1.24.2A shows the results for a single drop sort envelope and Table 1.24.2B for a 1.5 drop envelope (i.e., a purity mask of 1.5). Also shown are the numbers of wanted cells collected per hour. To calculate sort efficiencies for other input values (input rate, drop frequency, wanted %, and drop envelope) see *http://flowcytometry.med.unc.edu/sorting.htm*.

also aid in the reduction of clumping. Note that the addition of serum will negate the effect of the EDTA.

The media used in the preparation of the cells can also affect the cell viability. In general, the use of PBS for viable cell sorting should be avoided. Culture medium is preferred but optimally should be devoid of phenol red and other molecules (e.g., flavins) that can contribute to autofluorescence. For mouse lymphocytes for example, we have found that the use of RPMI-1640 is useful. Obviously, if one wishes to use a biotin–streptavidin step in the staining procedure, the medium must also lack biotin. An RPMI-1640 media without phenol red, flavins, and biotin is available as a custom order from HyClone (*http://www.hyclone.com*; cat. no. SH3A025.01). In lieu of culture media, but preferable to PBS, would be a balanced salt solution e.g., Hank's balanced salt solution (HBSS), again preferably without the phenol red unless you have tested and demonstrated that it does not interfere (see *APPENDIX 2A* for the recipe). Media containing a CO_2-carbonate buffering system should be avoided or should be buffered with a non-CO_2-based buffer, e.g., HEPES, pH 7.2 to 7.5. If not, the medium will lose CO_2 during the sort and become quite alkaline, which may be detrimental to the health of the cells. This is especially true when cloning into micro-well culture plates where a large surface to volume ratio accelerates the pH increase. The sample fluid should contain some type of protein. This may either be BSA or fetal bovine serum to a final concentration of 1% to 2% (and heat inactivated at 56°C for 30 min.). Serum should be filtered prior to adding to the media to avoid the introduction of any fibrin or other particulate matter into the sample. However, even with filtering, some sera will produce a "debris" population on the FSC/SSC plots and this could be taken as a poor sort result if not recognized.

The final concentration of cells in the sample will vary considerably depending on the number of cells available, the cell type, and its proclivity for clumping, and the desired

Table 1.24.2 Sort Efficiencies for a 100-μm Tip at a Sheath Pressure of 27 psi[a]

Input rate (cells/sec)	% wanted cells	Input rate of wanted cells(/sec)	Best sort rate (cells/sec)	Wanted cells collected per hour	% sorted	Sort efficiency (%)
A. 100 μm tip – 27 psi – 42,000 drops/sec – 1 drop sort envelope						
2500	20.0	500	477	1.7×10^6	19.07	95.34
5000		1000	909	3.3×10^6	18.18	90.91
10,000		2000	1,653	5.9×10^6	16.53	82.66
15,000		3000	2,254	8.1×10^6	15.03	75.15
2500	5.0	125	118	4.2×10^5	4.70	94.50
5000		250	223	8.0×10^5	4.47	89.31
10,000		500	398	1.4×10^6	3.99	79.76
15,000		750	534	1.9×10^6	3.56	71.23
2500	1.0	25	23	8.5×10^4	0.94	94.28
5000		50	44	1.6×10^5	0.89	88.88
10,000		100	79	2.8×10^5	0.79	79.00
15,000		150	105	3.8×10^5	0.70	70.22
2500	0.2	5	5	1.7×10^4	0.19	94.23
5000		10	9	3.2×10^4	0.18	88.79
10,000		20	16	5.6×10^4	0.16	78.85
15,000		30	21	7.5×10^4	0.14	70.02
B. 100 μm tip – 27 psi – 42,000 drops/sec – 1.5 drop envelope						
2500	20.0	500	465	1.7×10^6	18.62	93.11
5000		1000	866	3.1×10^6	17.33	86.69
10,000		2000	1,503	5.4×10^6	15.02	75.15
15,000		3000	1,954	7.0×10^6	13.03	65.14
2500	5.0	125	114	4.1×10^5	4.59	91.87
5000		250	211	7.6×10^5	4.22	84.40
10,000		500	356	1.3×10^6	3.56	71.23
15,000		750	451	1.6×10^6	3.01	60.11
2500	1.0	25	23	8.2×10^4	0.92	91.54
5000		50	42	1.5×10^5	0.84	83.80
10,000		100	70	2.5×10^5	0.70	70.22
15,000		150	88	3.1×10^5	0.59	58.84

continued

Table 1.24.2 Sort Efficiencies for a 100-μm Tip at a Sheath Pressure of 27 psi[a], *continued*

Input rate (cells/sec)	% wanted cells	Input rate of wanted cells(/sec)	Best sort rate (cells/sec)	Wanted cells collected per hour	% sorted	Sort efficiency (%)
B. 100 μm tip – 27 psi – 42,000 drops/sec – 1.5 drop envelope, *continued*						
2500	0.2	5	5	1.6×10^4	0.18	91.47
5000		10	8	3.0×10^4	0.17	83.68
10,000		20	14	5.0×10^4	0.14	70.02
15,000		30	18	6.3×10^4	0.12	58.59

[a]Sort efficiencies are calculated for a 100-μm tip at a 27 psi sheath pressure at various input rates and wanted cell frequencies. Table 1.24.3A shows the results for a single drop sort envelope and Table 1.24.3B for a 1.5 drop envelope (i.e., a purity mask of 1.5). Also shown are the numbers of wanted cells collected per hour. To calculate sort efficiencies for other input values (input rate, drop frequency, wanted %, and drop envelope), see *http://flowcytometry.med.unc.edu/sorting.htm*.

speed of sorting. Some cell types, e.g., lymphocytes, will tolerate the high concentration necessary for very high-speed sorting better than most other cell types. Cell concentrations in the range of 2 to 5 $\times$ 10^7/ml will facilitate sorting at rates up to 35,000/sec without undue increase in the core stream diameter, which should be kept small for best cell analysis. Lower cell concentrations will result in lower speed sorting as a consequence of the need to keep the core stream diameter small. We prefer to determine the optimum core stream diameter by running an alignment particle that has a concentration of 10^6/ml. The sample pressure is adjusted so that the CV of the test particles is optimal and the pressure noted. Cells are then run at that sample pressure or up to 0.3 psi higher. The sort speed is then entirely dependent on the cell concentration.

During the sort, cells will settle out in the sample tube effectively increasing the sample concentration at the bottom of the tube and increasing the threshold speed at the laser. This settling can cause a number of issues. The increase in speed, if not adjusted using the sample pressure, may exceed the conditions desired for sort recovery and purity. It may also lead to an obstruction of the sample pickup line at the end in the sample tube. This will be indicated by a loss in trigger rate that cannot be explained by a reduction in the cell concentration, and the trigger rate can become zero. The blockage will need to be removed by removing the sample tube and backflushing the sample line. This can result in a significant loss of cells if this needs to be repeated frequently, as all the cells in the sample line will be lost. Sorters should be configured with a mechanism for stirring the sample tube to help eliminate this. The tendency for this to happen is also likely a result of the sample preparation, as we have observed that the same types of samples may or may not have this problem. Careful attention to the details of the sample preparation can avoid this to a large extent. In general, adherent cells have a greater propensity to rapid settling than do cells that normally exist in suspension.

Usually the sample tube will be kept cold (e.g., 4°C), which helps to maintain viability especially over the course of long sorts. Temperature control is provided by a refrigerated recirculating unit, which is connected to the sample support device by insulated tubing. If possible, the temperature of the bath should be set by measuring the temperature at the sample tube to compensate for temperature increase over the transmission tubing. However, some cells may be negatively affected by cold and they may do better at room temperature. This should be established for each individual cell type.

Cell viability may of course be impacted during the sort due to a number of issues. When high-speed cell sorting first became available, many predicted that the cells would not survive the sudden decompression from a relatively high pressure to ambient pressure. This has not proved to be a concern for most cell types and at the pressures currently used in commercial cell sorters (i.e., about 60 psi as the upper limit). However, dyes to detect cell death should be included in the sample to help eliminate dead cells from being sorted. The use of propidium iodide at a final concentration of 1 μg/ml is commonly used; however, due to its broad emission range it is less attractive to investigators who want to sort using many different fluorochromes. DAPI at 0.1 μg/ml is a reasonable alternative as it can be easily excited with either a UV or violet laser, which

leaves many options available for other fluorochrome choices. TOPRO-3 can also be used off the red laser.

There are scattered anecdotal reports that cells (e.g., T lymphocytes or certain bone marrow cells) may have reduced viability or be viable but have reduced cell functionality (e.g., lowered responses to specific stimuli) following high-speed cell sorting. To our knowledge, this has never been rigorously tested and published. The experiences of others seem to contradict these to some extent. Users should always be prepared to test the viability and functionality of their cells after experiencing the sort conditions being used.

There are some clear indications that certain sort conditions can negatively affect the viability of the sorted cells. When the diameter of the nozzle tip is not properly matched to the size/shape of the cells being sorted, viability can decrease. As we discussed above, cells can affect the dynamics of droplet formation leading to irregular breakoff patterns and droplets of various sizes. This leads to side stream fanning. We have observed that when this occurs the sorted cells have a significantly decreased viability presumably due to shear forces at the drop breakoff. Thus, it is critical that the cell size and nozzle size be properly matched. Often this is not clear before hand and will need to be empirically determined by trying various tip diameters until one is found where the side stream fanning is reduced significantly or eliminated. As a rule of thumb, the nozzle orifice should be at least five times the diameter of the cell.

Most sorters use some form of PBS as the sheath fluid. The fluid must of course be free of any agents (e.g., preservatives or sterility maintaining components) that may damage the sorted cells. For some sensitive cell types, the PBS may need to be replaced with a more supportive medium e.g., HBSS or a culture media. (*NOTE:* If using a medium containing carbohydrates, the system must be purged of the media before shutting down the instrument, as the carbohydrates will promote bacterial growth in the fluidics system leading to subsequent bacterial contamination issues). Attention must be placed on maintenance of proper pH of this media as discussed above. Sorted cells must of course, be captured in some vessel, usually a test tube in the case of bulk sorting. Some type of fluid should be placed in the capture tube to prevent drying of the sorted material (in the case of very low frequency sort events) or to maintain viability. When using PBS as the sheath fluid, the capture fluid will become diluted by the PBS, and it will change concentration and this should be taken into account. It is often advantageous to include serum (or other protein e.g., BSA) in the capture fluid to improve viability. Some users sort into pure serum with the dilution factor from the sheath fluid in mind. This should be tested, as some cells may experience toxic effects from this.

The nature of the experiment will also dictate what type of collection fluid is used. The comments above have discussed collection fluids when cells are intended for re-culture or transfer into animals. Other experiments require different procedures. For example, when cells are sorted for recovery of RNA (or DNA), investigators sometimes prefer to sort directly into RNA isolation (lysis) buffers. These immediately lyse the cells and in the case of isolating RNA can contain RNase inhibitors. This approach is useful to limit the time and possibility of RNA profiles changing as a result of the sort manipulations. An example of a commercial reagent for this is RNAlater (Qiagen, *http://www1.qiagen.com*). One obvious problem with this approach, however, is that it is not possible to re-analyze the sort result.

During the collection of the sorted cells, the sheath fluid will tend to layer on top of the capture fluid. The sort should be paused frequently to mix the sort collection tubes, otherwise the cells will essentially be sitting in just sheath fluid. No sorter on the market has an automated device for performing this mixing. The inclusion of antibiotics in the capture fluid is not normally necessary. Most antibiotics require cellular metabolism and if the sorted sample is being kept cold, this will not occur to any significant degree. The sorted cells will usually need to be concentrated by centrifugation and the media replaced to remove the sheath fluid component of the collected sample anyway. The inclusion of antibiotics during the subsequent culture of the cells is recommended. Most sorters can produce sorts that are sterile and even without antibiotics will remain free of microbial contamination. However, the environments of most sorters are uncontrollable in regard to microorganisms and while the instrument can be sterilized (see below) environmental contamination especially by airborne contaminants can occur. The inclusion of a broad-spectrum antibiotic in place of or in addition to the typical penicillin-streptomycin antibiotic combination frequently used is advantageous. We have long recommended the use of gentamicin at 50 μg/ml for one week after the sort. In most cases, sorted cells are precious and loss due to contamination is costly

both in money and time and should be avoided by taking these precautions. The effects of any antibiotic on the sorted cells should be determined by each investigator.

Problems with Static Electrical Charge

Sort collection tubes made of polypropylene are preferable. The charged environment of the sort collection tube as it fills with sorted cell containing droplets can cause cells to adhere tightly to the walls of polystyrene tubes and the cells are difficult if not impossible to recover. This can lead to substantial loss of sorted cells. Alternatively, one can attempt to inhibit the binding to polystyrene by coating the tube with serum or other protein.

High-speed sorting provides the possibility (depending on population frequency) for the rapid accumulation of large numbers of sorted cells. A potential issue with the sort collection tubes is that of charge accumulation. When large numbers of sorted (i.e., charged) droplets are deposited into a collection tube, a substantial static charge can accumulate within the tube. It is large enough (although not at all dangerous) to provide a reasonable and noticeable shock if you discharge it through yourself. When the charge becomes large enough, it can actually cause sorted droplets to be repelled (due to like charge repulsion) out of the tube, resulting in lost cells. No currently produced sorter provides a means to discharge the charge. Use of polypropylene tubes can mitigate the effect to some extent and this coupled with the issue raised above about polystyrene tubes further supports the use of polypropylene tubes. If the charge issue in the sort collection tube is an issue, there are a couple ways to avoid problems. The first is to change collection tubes frequently such that the tube is only partially filled reducing the total charge and the likelihood that cells will be repelled out of the tube. The frequency of changing tubes can be reduced by using larger collection tubes when possible. Another approach that has been used is to insert a sterile (if necessary) platinum wire into each sort collection tube prior to beginning the sort. The other end of the wire is connected to a suitable ground point to discharge the charge from the sorted drops before it accumulates.

Instrument Setup

Each different brand of sorter and even each sorter of the same type will have its own idiosyncrasies for optimal sort performance and the operator will need to learn these for their particular instrument. In general, however, all sorters operate within a fairly tight range of specifications. The first step in preparing the sorter is sterilizing the system prior to a sterile sort. There are many variations of how this is performed and this may vary depending on the particular fluidics system for each type of sorter. Most current commercial sorters have adopted relatively straightforward fluidics systems that are easy to sterilize (probably more properly stated as disinfected). Most sorters have inline filters (0.22-μm or 0.45-μm) that will remove microbiological contaminates from the sheath fluid relieving the need to actually sterilize the sheath tank and sheath fluid, providing that the filters are regularly chemically sterilized. The most common disinfectants used are 10% bleach (i.e., a 1:10 dilution of the standard concentration of household bleach), 3% H_2O_2 (hydrogen peroxide), or 70% ethanol. Operators will need to establish the chemical compatibility of the filter and instrument components with the selected disinfectant. Check with your instrument manufacturer for recommendations.

The next step in preparing the sorter is preparation of the sheath fluid. Beyond what we have discussed above, the sheath fluid needs to be free of air bubbles. Some have recommended that a vacuum be applied to the sheath fluid to "debubble" the fluid but this is not usually necessary. The most frequent place where air bubbles are introduced is leaving the sheath fluid reservoir pressured for long periods of time e.g., overnight. If the tank is then depressurized, you will be able to see the release of millions of tiny air bubbles from the air that has been dissolved in the fluid. This is especially a problem where the tank is left pressurized overnight at 60 psi and then the same fluid is used the following day at a lower pressure e.g., 30 psi. In the event of leaving a system pressurized for a long period, it is best to discard and replace the sheath fluid. Sheath fluid may be purchased or prepared. PBS is very simple to prepare and inexpensive compared to commercial preparations with no disadvantages noted, but the commercial preparations do offer a significant convenience. Prepared PBS for the sorters should be filtered (by pressurization) through a 0.22-μm cartridge filter (can be used multiple times) to remove any particulates before being placed in the sheath fluid tanks. This extends the life of the in-line sheath filter substantially. Another source of air bubbles in the system is within the in-line sheath filter which can easily be dislodged by gentle tapping of the filter and bleeding off (under pressure) of the elaborated

Table 1.24.3 General Values for Drop Drive Frequency and Sheath Pressure for Various Commonly Used Nozzle Tip Orifice Diameters

Nozzle diameter	Sheath pressure	Frequency
50 μm	60-80 psi	120-160 KHz
70 μm	45-60 psi	65-100 KHz
80 μm	35-50 psi	45-80 KHz
100 μm	16-30 psi	28-45 KHz
130 μm	10-15 psi	16-25 KHz
150 μm	6-10 psi	7-12 KHz

bubbles. Non-pleated sheath filters, which are disposable 0.22-μm filter discs placed in a holder, can also be used.

The fluidics system of the sorter must be stabilized for drop formation and usually this requires the system to operate for 30 to 60 min prior to setting up the instrument, assuming there are no substantial issues e.g., system bubbles. The drop breakoff point is critical to the sorting process as described above and needs to be established. Operators will learn with time the approximate settings (drop drive amplitude and frequency) for each combination of nozzle tip diameter and sheath pressure, but some general guidelines are available. Table 1.24.3 provides some general values for commonly used nozzle sizes.

In general, the proper drop drive frequency will be one that provides the shortest or near the shortest breakoff. Note the shortest drop breakoff should be achieved with the drop drive frequency not with the amplitude. The amplitude—assuming it is set for the generally correct setting—should be tweaked to provide the best breakoff. The correct amplitude setting will provide the best side streams (see below). The quality of the drop breakoff is assessed by viewing the drop camera. The last attached droplet should be attached to the stream by a straight narrow neck of fluid. Satellite drops will form from the fluid comprising this neck. It is necessary that the satellite droplets fuse with a major droplet at some point from the breakoff (see Fig. 1.24.7). If this does not happen, the charged satellite droplets will usually contaminate the deflection plates interfering with the deflection field strength and resulting in side streams deflecting to incorrect positions. Theoretically, it is also best that the satellite fuse with the drop that generated it (i.e., the satellite should fuse to the top of the drops). This is because any charged/sorted satellites carry the same charge as the drop from which they derived. While technically correct, in practice there is little observed effect with today's sorters even if they fuse to the bottom of the drops. It is usually satisfactory if the satellite droplets fuse within 5 to 8 drop units from the breakoff. Satellite droplets fusing in 4 drop units or less usually indicates an incorrect drop drive frequency or amplitude resulting in too short of a breakoff. A very pointed, as opposed to long narrow fluid neck also indicates too short of a breakoff. Once the drop breakoff appears correct, the deflection plates may be charged and side streams observed. The amplitude (and/or frequency) may need to be changed slightly to provide tight side streams and, in some instruments, properly synchronize the charging electronics and the drop formation. The latter is visualized by the biggest deflection of the side streams. Different instruments have different approaches to this and the manufacturer's guidelines for setting frequency and amplitude should be followed.

At this point, the system should be optically aligned if necessary using alignment beads. This should be done before establishing the drop delay as vertical movement of the nozzle may affect the operator's reference point for maintaining the proper drop delay. Different instrument manufacturers have implemented different methods to establish the proper drop delay setting. All require the use of some brightly fluorescent particle. Some manufacturers require the use of fluorescent particles (e.g., FlowChecks) and the observation of sorted particles in a fluorescent microscope. Sort gates are established for the particles (using either fluorescence or light scatter) and these are directed to a single sort stream. The system attempts to sort a defined number of particles to a sort stream and the sorted

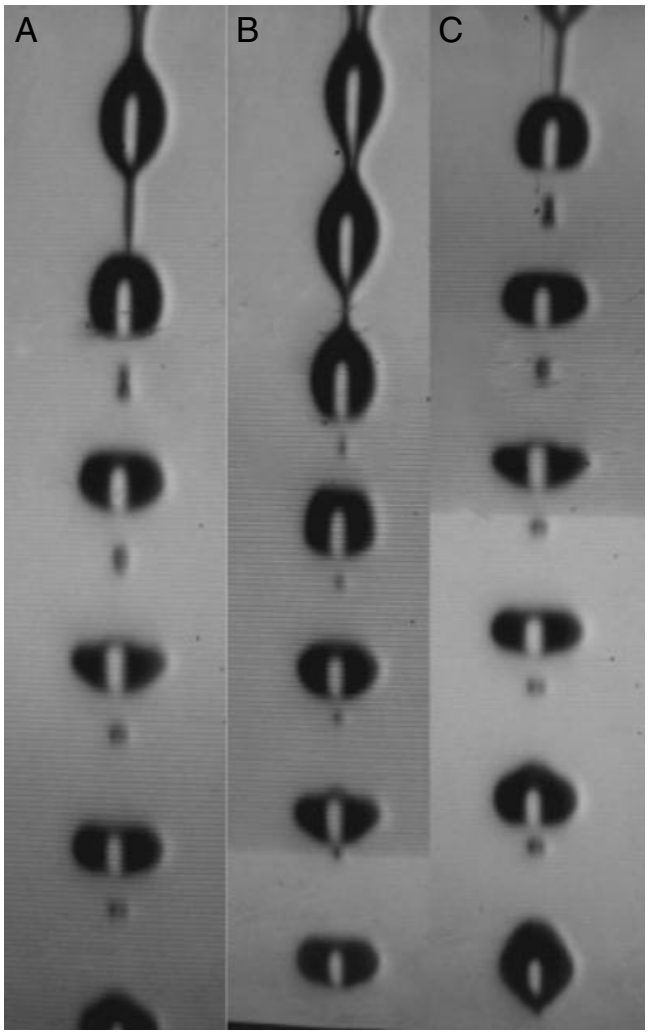

Figure 1.24.7 Shown are an example of a good drop breakoff (**A**) and a poor breakoff (**B**). In panel B, the drop drive amplitude and/or frequency are set too high. The neck between the last and second to last attached drops is too short. Compare this to the neck in panel A, which is long and straight. Also visible in panel B is that the first satellite drop is too small and the satellite drop fuses back with the main drops within 3 drops which is too fast. Panel **C** shows how the satellite drops fuse back at drop 5 with a good breakoff. Also, note the slightly longer first satellite when the drop breakoff is good.

particles are deposited in a puddle on a microscope slide. Several puddles are produced by automated or manual advancement of the slide simultaneously with automated or manual increase in the drop delay in full drop increments. The puddles are observed under the microscope for the presence of fluorescent particles. The puddle containing the particles is associated with a particular drop delay setting. Two adjacent puddles may contain particles indicating that the drop delay needs to change in fractional drop units. When all the particles are contained in a single drop (or within a very small percentage <4%), the proper drop delay is established and is either automatically entered into the system by the computer or manually set. The user should refer to the specific documentation for their instrument for complete details for their particular instrument.

Other systems use an image-based system. Here the use of special beads that excite with long wavelength red laser light are required. A forward-scatter sort gate is drawn to include all the beads and sent to be deflected to the left or right sort stream. A separate laser illuminates the waste and side stream and the fluorescence from the particles is viewed in a CCD camera. An optical filter is dropped in front of the camera to allow only the fluorescent light of the illuminated bead images to be

seen. As the drop delay in increments of full drops or tenths of a drop is changed, the operator can observe a shift of the particles from the waste stream to the side stream. When the maximum number of fluorescent bead flashes are seen in the side stream and a minimum are seen in the waste, the proper delay is achieved. Newer instruments automate this process with image analysis.

A new system uses a similar approach but the there is no need to observe the side streams. The main stream is illuminated with a laser at the drop breakoff point of the stream. This laser flashes on and off at the input drop delay (i.e., comes on *x* drop delay units after the particle is observed in the main laser) and the position of the fluorescent particles is observed. The system will either operate manually or automatically. The drop delay is changed and the position of the fluorescent particles will change. The proper drop delay (actually the drop delay minus 1 drop unit) occurs when the majority of the particles are in the first detached drop (i.e., the minus 1 drop delay puts it back to the last attached drop). Fractional drop delay units are then input to make all particles appear in the same drop. The automatic system calculates the fractional drop delay setting for the user.

Once the drop delay is established, the drop breakoff point can be maintained either manually or automatically. The automatic systems rely on image analysis of the image from the breakoff camera and feedback loops. Thus, a high-quality image is required. To obtain this image quality may require adjustment of the camera (e.g., focus) or the illumination of the stream. These adjustments may be performed by the operator or by the company service representatives. Manual adjustment is made by reference to the drop breakoff image by, for example, drawing a reference line on the image monitor at the edge or middle of the last attached drop. The drop breakoff may change due to a loss of efficiency in coupling the acoustic energy from the piezoelectric crystal to the fluid stream or in other ways, e.g., a change in the temperature of the fluid. Also, the position of the drop breakoff may change due to a change in the stream velocity, e.g., associated with a drift in the sheath pressure. This can occur during long sorts when the level of the sheath fluid in the sheath tank decreases, decreasing the effective head pressure component of the sheath fluid pressure. In the former two cases, the image change noted will be a drift to where the last attached drop either begins to become detached or the length of the first satellite drop begins to increase towards connecting the last attached drop and the first detached drop. In both of these cases, the drop drive amplitude should be decreased or increased, respectively. If the total length of the stream drifts—observable by a change in the position of the last attached drop relative to the reference line on the monitor—the amplitude should not be adjusted. See Figure 1.24.8.

Multiple Sort Streams

The ability to sort, at high speeds, more than one population of cells has enormous impact on sorting productivity. This reduces not only the time required, but also the amount of sample needed. All instruments currently in production permit the simultaneous sorting of up to 4 populations and a new sorter in development is reported to be able to sort 6 populations (see Fig. 1.24.2). The sorting of 4 populations is implemented by providing two stream charging amplitudes in each direction—positive and negative. The primary issue in multi-stream sorting is in providing the necessary stream separation to direct each stream into a suitable-sized collection vessel. The stream separation issue has required the redesign of the deflection plates to "shape" the charge, as well as increasing the amplitude of the stream charge and the voltage applied to the deflection plates and developing electronics that can better apply the charge to the stream to ensure a full charge is applied. The application of the phase mask (or single mode—see below) can improve side stream integrity when necessary. By restricting the sorting to drops where the cell is in the middle part of the drop, drop size variation due to cell perturbation of the breakoff is reduced. However, this mode will result in a higher frequency of sorting aborts and, thus, loss of wanted cells. Multiple stream deflection requires that all components of the sorting process be optimized. The relative closeness of the streams requires that side streams have little fanning. This requires the operator to optimally synchronize the charging electronics with the droplet breakoff. The drop drive amplitude must also be properly adjusted and maintained. Satellite droplets **must** fuse with the main droplets or the deflection plates will wet. The nozzle tip diameter and the cell size/shape must be coordinated so that droplet formation is perturbed as little as possible. Cell debris must also be minimized.

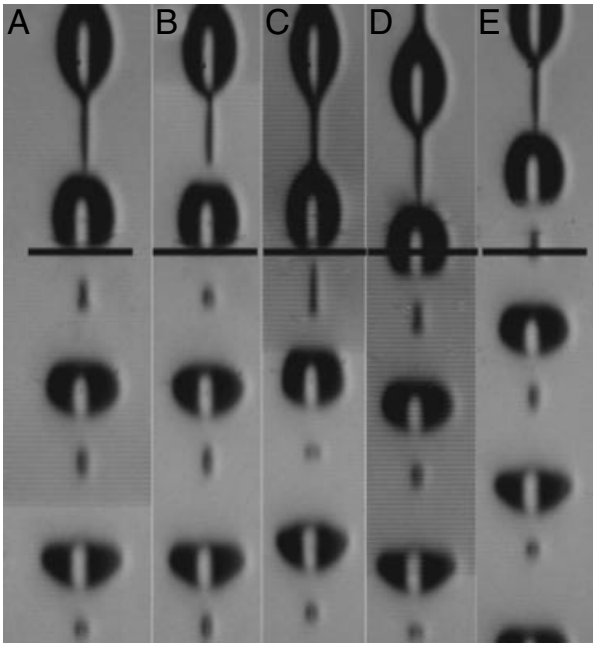

Figure 1.24.8 Shown here are photographic images from the drop breakoff camera demonstrating the drop breakoff changing over time and how to adjust to return to a good breakoff. The horizontal line is a reference point on the monitor. Panel **A** shows a good starting breakoff. Panel **B** shows that the breakoff is creeping shorter and the desired last attached drop (in panel A) is now separating from the neck. Panel **C** shows how the breakoff is beginning to creep longer as demonstrated by the lengthening of the first satellite drop, as well as the last attached drop. The reference line on the camera monitor shows that although the drop breakoff is changing, the length from the nozzle has not changed. The breakoff change represented by the images in panels B and C should be corrected by decreasing or increasing the drop drive amplitude, respectively. Panels **D** and **E** show the effect of increasing and decreasing stream velocity, respectively. While the breakoff pattern looks the same as in panel A, the position of the last attached drop has moved down or up from the reference line. These types of changes should not be corrected by changing the drop drive amplitude.

Sort Strategies

The overall strategy one takes for a particular sort depends on the intended outcome. The sorting of relatively rare cells is an example. If relatively large numbers of these cells are needed, one must process very large numbers of starting cells. A single pass flow sorting approach to this to give high purity will likely mean very long sort times to process the large number at speeds that permit relatively high purity. Pre-enrichment strategies can be employed. These can involve non-flow cytometric sorting approaches (e.g., magnetic separation) or flow cytometric sorting approaches. Depending on the application, approaches using, for example, magnetic sorting are often best done by a negative selection strategy. If there is a need for sorting on multiple markers simultaneously, these approaches may not be the best. One must always evaluate the cost and yield of these processes to determine which is best for a particular application. The flow cytometric approach to this problem is to first do a very high-speed enrichment sort followed by a slower speed, high purity sort. The speed of the enrich sort will be determined primarily by the type of cells (in the whole sample not just the desired cell type) being sorted. For lymphocytes, these pre-enrichment sorts could be done in the 30,000 to 70,000 cells per sec range. The intent of the enrichment sort is to sort every desired cell and increase the purity, but recognizing that the purity will be substantially below that acceptable. The sorted cells from the enrichment sort are concentrated (by centrifugation), and then put back on the sorter and further processed using a much slower speed purity sort. If two sorters are available, as pre-enriched cells accumulate these can periodically be transferred to the second sorter to begin the purity sort further reducing the time required to complete the total sort.

A second strategy, which works to increase the purity of the rare sort populations is to stain

all cells with some marker and then include both inclusive and exclusive gating strategies. For example, when looking for very minor subpopulations, which can only be identified with one or a few markers, some of which may also be expressed on other cells of non-interest, it is helpful to include some markers that would stain the cells of non-interest and gate these cells out prior to establishing inclusive gates. This not only makes it easier to identify the cells of interest, but by providing more criteria from which to sort this will naturally improve the purity of the sort. This can easily be achieved with only one fluorescent channel usually referred to as a "dump" channel since everything that is not your population of interest will be excluded by positive staining in this channel (i.e., only the negative cells will be included in the sort gating strategy). A viability dye, as well as antibodies to identify the cells of non-interest, can all be identified with fluorochromes that emit in the same channel. To simplify the antibody staining even further, antibodies can all be conjugated to biotin and then one fluorochrome conjugated to streptavidin can be used to identify all of their staining. It is imperative that the compensation control contain the same combination used in the dump channel.

Sorting on only a single fluorescent channel, such as sorting for the expression of a single fluorescent protein (e.g., GFP), can also present challenges and requires specific strategies to obtain good results. In this case, it can be helpful to use a second parameter, as opposed to a histogram, to identify the positive cells, unless the populations are completely resolvable. For instance, using side scatter as a second dimension for plotting the single fluorescence parameter can be helpful in resolving a dim population, which shows no resolution on a histogram. Another strategy that is particularly useful for fluorescence that falls in the range of autofluorescence (usually the lower wavelengths) such as GFP, FITC, PE, or YFP, is to use a second "empty" channel that is closest in wavelength and compensate out the true fluorescence from the autofluorescence channel (Roederer and Murphy, 1986; Alberti et al., 1987). This will help to avoid contribution of autofluorescent negative cells within the sort gate (see Fig. 1.24.9). This strategy works best

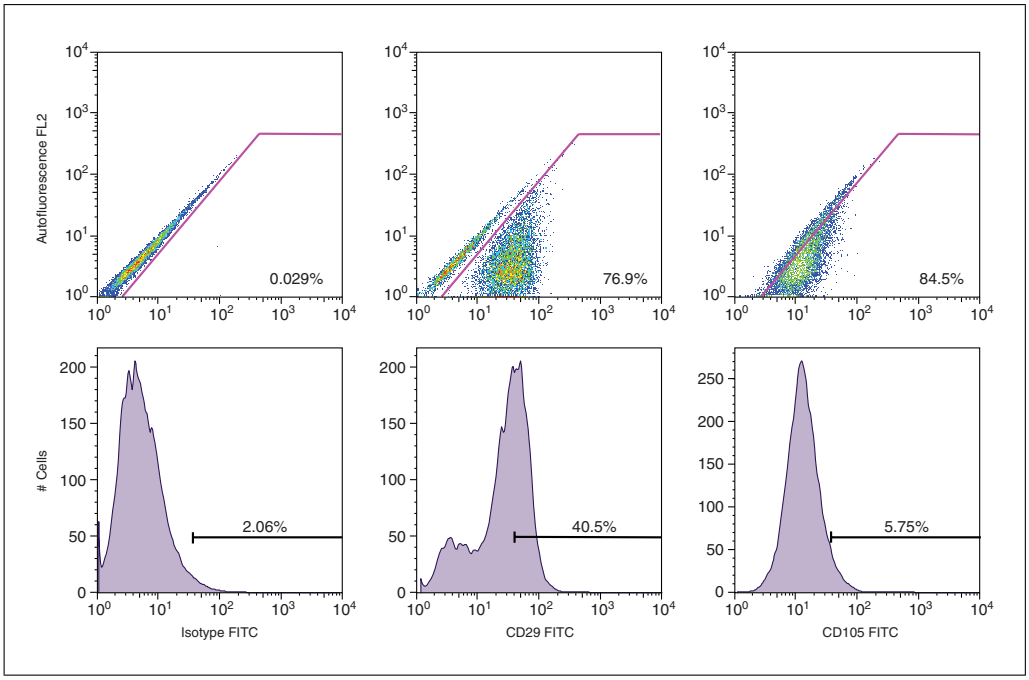

Figure 1.24.9 The data plots shown illustrate the utility of adding an autofluorescence parameter to resolve negatives and dim positives. In this case, the cells were single-color stained with two FITC-labeled antibodies (CD29 or CD105) (or isotype control) and plotted vs. FL2 which was autofluorescence only. The fluorescein spillover into FL2 was compensated. The left two plots show the isotype control and the gates that were set by visualizing this "negative" control sample. If the data had been visualized using just a single-parameter histogram (bottom row), it is difficult to tell where the positives separate from the negatives. The percent positives are grossly underestimated by the simplistic method using the single parameter plots. The two-parameter plots easily provide resolution of the positives. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cy0124*.

for cells with high levels of autofluorescence (cultured cell lines, macrophages, fibroblast, highly granulated cells, etc.) and is not as useful with relatively non-autofluorescent cells such as lymphocytes. The second most important thing in this situation is to eliminate aggregates with a doublet discriminator (i.e., either area vs. width, or area vs. height), as a negative cell stuck to a positive cell of interest may cause a cell to be sorted as a cell of interest and decrease the purity of the sorted population.

When samples contain debris (especially significant amounts), the operator must make a decision about how to deal with the debris. If the trigger channel threshold is set low enough to detect the debris, the debris events, when they contaminate a desired sort event, will cause that sort event to be aborted leading, in the case of substantial amounts of debris, to a substantial loss of cells that should have been sorted. The operator may choose, instead, to set the trigger channel threshold high enough to threshold out all or much of the debris. This will preserve the capture of the cells to be sorted, but will lead to a contamination of the sorted cells with debris. If the debris will not cause a problem for the subsequent use of the cells, it will not matter. The best solution, of course, is to remove the debris from the sample prior to the sort.

Avoiding Contamination between Samples

When multiple samples are sorted sequentially, one must consider the issue of cross-sample contamination. This is especially important when the sorted cells will be put back into long-term culture or used for PCR analysis as any contaminant will be exponentially (log base 2) expanded. This may be emphasized even more when cells are re-cultured, as some sorted populations may have a growth disadvantage compared to the contaminant cells. Two approaches are possible. One is to completely change out, between samples, the entire sorter components that contact the sample. This would include all the sample line tubing and the nozzle body and tip. On all the sorters currently in production this is either not possible, difficult and time consuming, and although possible on one commercial instrument is expensive. Most sites will not adopt this strategy. The other is to try to clean between samples. This is possible to do and we have done many sequential sorts where we could determine if a contamination had occurred and have never had one by using the cleaning method we have developed. However, one must always be aware that such cross-contamination could occur and warn investigators that they should check, if possible, to determine if cross-contamination has occurred. In circumstances where the nature of the cells precludes this determination, extraordinary measures need to be used to try to avoid the problem. The lower the cell density of the samples, the lower the probability of cross-contamination occurring. In addition, the operator should evaluate the sample path and remove, when possible, any components (e.g., valves or other tubing fittings) where cells could hang up and persist. Following the completion of a sample, the sample line is extensively back-flushed—e.g., 3 to 5 min. A chemical sterilant is then put on as a sample, the sample is boosted for 30 sec, and the chemical flushed through the line at the regular sample pressure for 5 min. We have typically used Exspor, a chlorine-based sterilant (no longer available), or 10% bleach (i.e., 0.525% to 0.6% chlorine based on standard bleach concentrations). The chemical sterilant volume should be sufficient to immerse the sample probe in the sterilant at least as far up the probe as the probe contacted the cell sample and should be more to eliminate any sample that splattered higher. Following the chemical sterilant, the sample line is again back-flushed for 3 min to remove the chemical. Attention must be paid to wash the external surface of the sample probe as the back-flushing only washes the internal surface. Lastly, the probe is flushed with sterile distilled water for 3 to 5 min (following a short boost).

Re-analysis of Sorted Samples

It is best if all sorted samples are re-analyzed to confirm that the sort produced the desired result. This should be requested by the investigators as a part of doing good science but is also necessary for the core facility to track performance issues. This is especially important in a large multi-user core facility where many types of samples are used. This allows for adjustment of procedures to accommodate different requirements for different cell types and applications. For investigators who sort the same sample types repeatedly and have good experience, this is probably less important but does not guarantee that the results from any given sort were satisfactory.

Sorting of Small Numbers of Cells (Cloning)

High-speed sorting is a particularly good process for isolating very small numbers of cells and placing a defined number of cells (as few as one cell) into small receptacles e.g., tissue culture cluster wells and PCR tubes. All instruments currently in production support this application and can deposit cells into wells of plates containing from 6 to 384 wells (some will also do 1024-well plates). Cells may also be deposited onto other surfaces e.g., slides, or nylon membranes. The setup of the instrument can become critical especially when small diameter wells in plates with more than 96 wells are used as the target area to deposit the cells. When performing cloning, only a single population can be sorted at one time. So, if multiple populations from a sample are required, multiple suitably sized aliquots of the sample will be needed for each population to be sorted. The instrument configuration that provides the most precise position targeting of the sorted cell is one in which the trajectory of the sorted cell is straight rather than deflected as is done in normal sorting. In this configuration, the waste stream is deflected requiring repositioning of the waste catch tube, as opposed to normal sorting where the sorted drops are deflected at some angle away from the central waste stream. In this approach to cloning, the drops containing the desired cells are not charged and they follow a straight down trajectory. This prevents the cells from hitting the sides of targets that have a small cross-sectional area and are relatively deep (e.g., 384-well plate wells). Not all instruments in use provide this capability or have the ability to allow the user to modify the system to do this. Instruments using the deflection approach may be less efficient at having one cell actually deposited in every well for at least certain plate configurations.

When targeting wells, particular attention must be paid to the alignment of the plate wells with the sorted stream. Small misalignment may result in some number of cells not being deposited in the wells. This is especially important as the cross-sectional area of the target well becomes increasingly smaller. It is also important to consider the cross-sectional area of the fluid in the well. If the well is a tapered well, as most PCR tubes are, then placement of small volumes of fluid (typically 4 to 10 μl) in the tapered bottom of the well provides a much smaller target than simply considering the cross-sectional area of the top of the well. The instrument hardware configuration and software should provide an easy way to visualize the location of targeting droplets and provide fine adjustment of the plate position. The stability of the plate support and movement systems must be excellent.

As discussed above, the sort mode used when depositing a precise number of cells must be the "single" sort mode. This mode ensures that only a single cell will be deposited with each sort drop even if the drop contains two wanted cells. Some instruments also include the ability to abort the sort even when the drop contains only 1 cell based on where in the drop the electronics expects the cell to occur. When cells occur in the central portion of the sorted drop, they are most likely to actually be in that drop and be recovered. When cells are at the edges of drops, there is a probability that the cell may actually partition to the neighboring drops and not make it to the sort target.

When sorting cells into culture plate wells where the intention is to grow out the cells, particular attention should, of course, be paid to cell viability. An important consideration in this regard can be the pH of the media placed in the well. As discussed above, small volumes of CO_2-carbonate buffered media will quickly become very alkaline outside of a CO_2-incubator. The media should be buffered with a strong non-CO_2-based buffer e.g., HEPES. In addition, as discussed above, antibiotics, which includes a broad-spectrum antibiotic, e.g., gentamicin, should be added to the media to combat bacterial contamination.

SAFETY

Laser Safety

As most high-speed cell sorters use lasers with relatively high laser powers, the potential for laser related injuries, particularly to the eye and skin, exists. The potential for injury varies with both the power and wavelength of the laser beam. For reference, the maximum safe power for a laser pointer is 5 mW. Most instrument manufacturers fully enclose the lasers during routine use to minimize the exposure and have safety interlocks in place that are intended to shut off the lasers when protective covers are removed. However, certain routine procedures, such as laser alignments, may require the defeat of such interlocks. It is important that all operators familiarize themselves with the potential hazards and risks of working with such lasers and employ recommended

safety procedures, such as labeling lasers with specific warnings, and wearing laser safety goggles when operating lasers to minimize the potential of exposure and biological damage. Operators should refer to published standards of laser safety such as ANSI Z136 in the U.S. (*http://www.z136.org*) and IEC 60825 internationally (*http://www.iec.ch/*).

Electrical Safety

In addition to the ionizing radiation effects of lasers, there also are the potential risks associated with many high-voltage exposures on high-speed cell sorters. On instruments with larger, higher powered lasers in the 1- to 2-W range, large high-powered power supplies are used which may pose lethal electrical hazards. Appropriately licensed personnel should install proper power sources. Of additional concern are potentially dangerous high voltage and current levels that may exist in many of the components of the electrical system including printed circuit boards. Large volt potentials exist across the deflection plates when turned on and serious electrical shock can occur if touched during operation. The operator is cautioned to pay close attention to both the indicator lights and software indication of the status of the deflection plate charging. Another source of potential exposure to voltage is the stream charging wire and nozzle. Operation of a high-speed cell sorter requires careful attention to the interactions with the system and total awareness of potential exposure to electrical shock.

Biosafety

Sorting of unfixed cells can present numerous sources of potential biohazardous exposure. Sample handling, aerosol generation, equipment maintenance, and waste management all pose potential risk. Samples, as well as sheath fluid, are under conditions of high pressure and, hence, can be a source of accidental exposure. Although the newer generation of cell sorters has gone to a design of sample containment within a closed unit, some of the older cell sorters that operate under conditions of high pressure are not equipped in this manner and the operator may be at risk if the sample tube blows off causing a splash. High-speed cell sorters are also known to produce a significant amount of aerosols through the formation of the droplets to be sorted. It is widely accepted that a major source of laboratory-acquired infection is through the generation of aerosols (Biosafety in Microbiological and Biomedical Laboratories, 5th Edition, *http://www.bmbl.od.nih.gov/*). Infectious aerosols are of particular concern, as they are dispersions of droplets that contain particles capable of causing infection (viruses, microbial agents, fungi, etc.). This hazard exists in jet-in-air instruments, as well as in instruments that combine a flow cell and jet-in-air sorting. Under normal operating conditions, high-speed cell sorters produce droplets in the range of 40 to 200 μm depending on pressure and drive frequency (Kevin Holmes ISAC Biosafety Workshop *http://www.isac-net.org/content/view/743/46/*) with satellite droplets in the range of 3 to 7 μm (Schmid et al., 2007). The larger droplets generally settle pretty quickly and do not pose a great level of risk. However, during sorting failure modes, where a nozzle may become clogged and the sort and waste streams may deflect away from the receptacle, impact on hard surfaces, and generate fine mists of droplets under conditions of high pressures, droplets of various undefined sizes can occur (Schmid et al., 2007). Recent studies have characterized these as being 0.5 to 20 μm, with the majority being between 1 to 5 μm (Kevin Holmes ISAC Biosafety Workshop *http://www.isac-net.org/content/view/743/46/*). This droplet size coincides with the known size range of particle absorption within the respiratory tract. (Schmid et al., 2007). As a result, it is imperative that efforts are made to minimize the exposure of the operator to these aerosols by active measures of aerosol containment within the instrument. Most manufacturers of current equipment provide some form of aerosol management, whether it be through the use of specific aerosol containment devices that are part of the instrument or by containing the entire instrument within a rated biosafety cabinet. The cabinets can be either Class I (only the operator is protected, the sample may be exposed to non-sterile airflow) or Class II (both the operator and the sample are protected by HEPA-filtered air) devices. Since none of these engineering devices can offer 100% protection from exposure to aerosols, it is advisable to supplement them with Personal Protective Equipment (PPE) such as Powered Air Purifying Respirators (PAPR) (Perfetto et al., 2003). Detailed evaluation and procedures for dealing with these biohazard risks can be found in multiple publications (Schmid et al., 1997, 2007; Oberyszyn and Robertson, 2001; Perfetto et al., 2003, 2004; Lennartz et al., 2005), as well as in *UNITS 3.3*, *3.5*, *& 3.6*.

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