

Sorting Theory

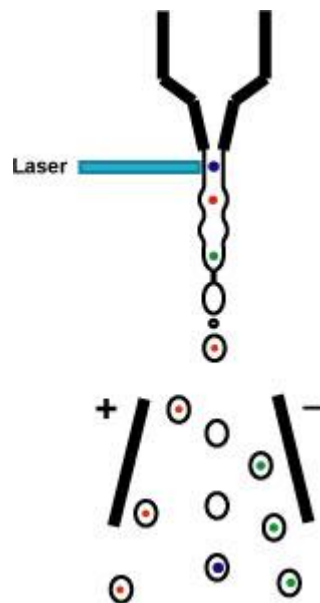
Sorting Concepts

Sorting is the process whereby cells of interest are separated into a collection vessel. All or our sorters, as well as all now available, can sort up to 4 populations of cells simultaneously. Sorting, of course, requires a first step of flow cytometric analysis to identify the cells or sorting. This process is, for all intents and purposes, identical to that performed in an analysis only instrument. All those components that make for good analysis are required for good sorting including compensation controls. Sorting can provide viable and sterile sorted cells in high purity and at relatively high recovery and speeds. The losses involved in sorting can vary greatly but all sorting is expected to have at least some loss of cells in the process as does any purification process. These issues will be discussed below.

Sorting, in essentially all current effective sorters, is performed by a process called electrostatic droplet sorting. While mechanical sorting is available (e.g. B-D FACSCalibur) it is at least 2 orders of magnitude slower than electrostatic droplet sorting. Attempts to build effective microfluidics sorters (sorting on a "Chip") while intriguing are not yet available commercially. In electrostatic droplet sorting a stream of fluid containing the particles to be sorted is ejected from a nozzle tip. The fluid stream is broken up into droplets by applying an acoustical energy to the fluid using a piezoelectric transducer. Precise control of the droplet formation is controlled by the instrument electronics and involves the frequency (clock) and amplitude of acoustical energy. Also a critical factor is the size of the nozzle orifice (i.e. stream diameter) and the pressure that the fluid stream is under. The relationship of these is described by the Rayleigh formulae. The cells are randomly distributed in the fluid stream and, thus, are partitioned in the fluid stream and, therefore, into the sorting droplets in a random fashion. The speed and purity of the sorting process are a function of this partitioning and how well the instrument can resolve the cells. For best purity the cells, on average, should be separated from each other by at least one droplet interval. Thus, to sort faster, i.e. to resolve more cells into droplets per second the droplet number generated per second (acoustical frequency) must increase. To increase the frequency and maintain precise droplet formation within a useful distance from the laser interrogation point the amplitude and fluid pressure must also increase. Thus, to sort faster we need to generate more droplets/second and use increasingly higher sheath fluid pressures and smaller diameter orifices. For the two most frequently used nozzle sizes - 70 and 100 μ m, sheath pressures are in the range of 45-60psi and 25-30psi, respectively. Thus, it should be obvious that we can sort faster with the smaller diameter fluid stream using higher sheath pressure. However, other factors discussed below also impact on the sorting and especially in the choice of nozzle size. The viability of cells, while certainly variable between different cells types, may be affected by increased sheath pressure and the rapid decompression which results when the cells exit the nozzle. However, this effect has generally been over estimated. The fluid stream and droplet generation must remain very stable over the length of the sort in order to maintain high purity and recovery. Current sorters incorporate image analysis feedback systems that can control, to some extent, the stability of the droplet generation.

To sort a cell the droplet containing that cell is charged either positively or negatively (and for 4-way sorting two amplitudes of each). The only way to provide the charge (i.e. to have an electric connection to the

charging circuit) is through the fluid stream. Thus, obviously, we need to use a sheath fluid which is capable of carrying an electric current - an isotonic saline solution suffices. The critical element in the process is that we must apply the charge just as the droplet containing our cell of interest is breaking off from the fluid stream. That droplet will have a connection to the electric circuit to permit it to be charged and the application of the charge will persist until the droplet detaches from the fluid stream. The separated droplet will then retain the applied charge since it no longer has a connection to the electric circuit - i.e. to ground. The droplet then falls between two charged plates - one negative and one positive. The electric field created will then cause the charged droplets to deflect toward the plate with the opposite charge from the droplet with the amount of deflection being proportional to the amount of charge carried by the droplet (also a function of the size of the droplet) and the amplitude of the deflection field. Test tubes placed in the appropriate place simply catch the falling droplets.



There are two critical aspects to the process other than the stability of the fluid stream and droplet formation discussed above. The first concerns the timing of the applied charge. As shown in the accompanying figure, the interrogation of the cell (i.e. when the cell is in the laser) must obviously happen first so the computer can decide if the cell matches the criteria established for sorting a cell. This process must take place for jet-in-air sorters (laser beam strikes the cell in the fluid stream just after it leaves the nozzle tip) before the stream begins to undulate as a precursor to droplet formation as this would disturb the laser beam. Since the cells are moving (at a sheath pressure of 60psi the speed of the fluid stream is approaching 30 meters/second) some time will elapse while the cell is traveling from the laser interrogation point to when it is in the last attached droplet. This is termed the drop delay. The units of the drop delay are the number of drop periods. The instrument operator either manually or with computer assist systems must determine precisely the drop delay and this time interval must be maintained very precisely for accurate sorting. Three of the sorters on the market each use a different approach. The Beckman-Coulter sorters (MoFlo and MoFlo XDP) require a manual determination of the drop delay. A specific number of fluorescent beads are sorted into individual puddles on a microscope slide (usually 100 or 160). For each puddle the operator or computer varies the time delay in whole drop increments. The sorted puddles are then inspected for the presence of sorted beads. The

puddle containing the most beads is determined. An adjacent puddle may also have some beads. The drop delay is then varied by fractions of a drop delay until all the sorted beads appear in a single puddle. The MoFlo is capable of dividing the drop delay (actually a single cycle of the drop generating frequency or clock) into 16 divisions. The MoFlo XDP can divide the drop drive period into 1,000 divisions. The B-D FACSAria uses a system called Accu-Drop. In this system special fluorescent beads are put through the sorter. The sorted and waste streams are illuminated with a laser beam with wavelength matching the excitation of the beads. Sorting is then started and the drop delay is varied while the operator watches the fluorescent image spot from the beads. Initially the beads will all be in the waste stream but when the delay is correct the bead flash will be predominantly in the sorted stream. The iCyt Reflection uses an automated bead-based image analysis system to identify when the fluorescent beads appear in the last attached drop relative to when they were in the detection laser.

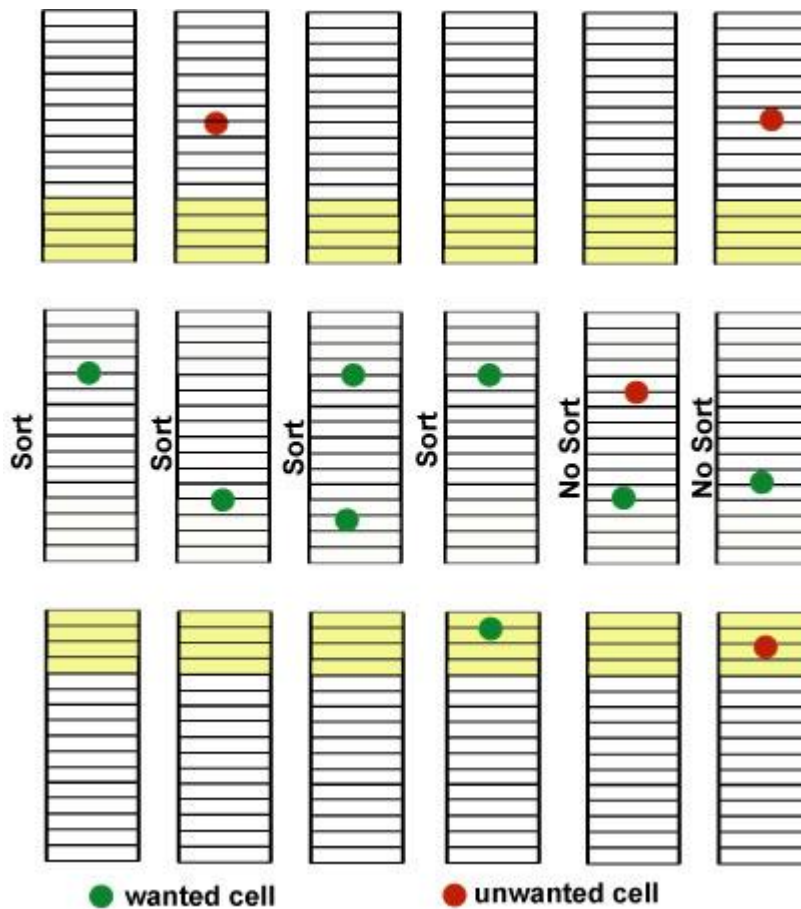
The droplet charging system also needs to vary the charge pulse precisely to the droplets. Droplets going into a single sorted stream must all have the same charge in order to maintain a tight sort stream that will hit the collection tube. If this does not happen the sort streams will "fan" out causing issues with cell-containing droplets missing the appropriate collection tube or even perhaps entering the incorrect sort collection tube. This charge amplitude needs to vary in response to the interval between sorted drops with the same charge or with different charge. For instance, if two cells to be sorted into the same stream are in adjacent droplets the charge amplitude to the second drop will need to be slightly different (less) from the first droplet. Thus, the computer must not only keep the timing interval constant but must also constantly be ready to vary the sort charge amplitude as the sort conditions vary. The size of the drops may also cause the side streams to fan. Cells (or debris) falling at drop boundaries perturb the drop formation leading to different sized drops. If the drops are different sizes then they will carry different amounts of charge and follow slightly different trajectories through the deflection field causing sort stream fanning. This needs to be avoided for clean sorting and best cell viability. Fanning of side streams indicating drop formation perturbation correlates with lowered viability of the sorted cells. This is generally thought to be a result of shear forces that act on cells at drop boundaries. This condition can occur when nozzle size (and resultant drop size) is not properly matched to the cell size and shape. Larger cells require a larger nozzle. In general, we prefer to use a nozzle size that is several times larger than the cells being sorted. We usually will use a 70 μ m tip with lymphocytes and lymphocyte sized cells. Most cultured, adherent cells will require the 100 μ m nozzle tip and some could require larger. Also, large amounts of debris in the sample, even though a debris particle is smaller than the cells, can cause problems with the way drops form leading to sort stream fanning, lowered viability, and uncertainty in the wanted cell actually being sorted. Note that as the tip size increases sorting speeds will need to decrease as larger tips require lower sheath pressure and generate fewer droplets per second (see statistics discussion below).

The sorter is able to estimate where in time a cell will occur relative to the drop periodicity. As noted above the MoFlo XDP can estimate to within 1/1000 of a drop period where the cell will occur. This is critical to knowing where cells are occurring relative to each other so the sorter computer can make decisions/predictions about how close the cells are to each other and to decide whether they can be separated to provide high purity. The sorter can be instructed to operate in several modes that affect the purity and recovery of potential sorted cells. For highest purity decisions can be made that will cause a sort event to be aborted if the cell to be sorted is too close to another cell that does not fit the sort criteria. Note

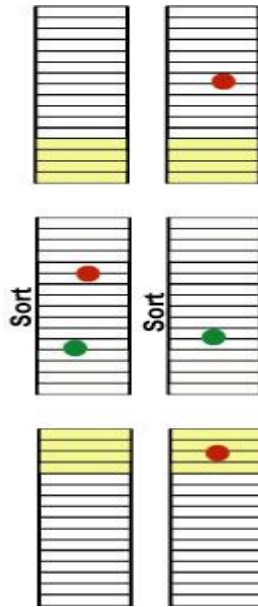
that the decision is made while the cell is in the laser beam part of the fluid stream and is made relative to the "clock period" of the droplet frequency. Thus, the computer can calculate where cells are relative to the droplet break off point and in fact in which drop they will occur and even in which part of a drop they will be. However, there is always a degree of uncertainty as to which drop a cell will partition into when cells occur near drop boundaries. These decision making processes are discussed below in more detail.

During sorting (and analysis) particles arrive at random at the interrogation laser(s) and, in sorting, at the last attached droplet where the charging occurs. The laser position and the drop rate and position of the last attached drop, however, are constant (or we hope they are or we have bigger problems). The probability for 1 or 2 (or more) particles arriving at the last attached droplet is described by the Poisson statistic ([see below](#)). The probability of more than 1 cell in a droplet is a function of the number of droplets being generated per second (drop frequency) and the number of cells entering the analysis stream per second. The tables below shows estimations of the frequency of the number of cells at various drop and cell input frequencies.

The ability to know where cells are relative to nearby cells provides the flow cytometer operator the ability to make decisions about how conflicts should affect the sort process. The decisions will affect purity and recovery. Also since cells can arrive at random relative to the drop frequency, i.e. the cell may be in any part of the drop, it is useful to be able to make sort decisions based on this prediction. If a cell arrives very near a drop boundary it is difficult to predict which drop it will actually partition into and as we will discuss below may actually perturb the drop break off leading to unpleasant results. Flow cytometry sorters have evolved to deal with these potential conflicts. The term used to describe these solutions are called sort modes or sort masks. Also in the early days of sorting the electronics were not fast enough to allow these predictions and so because of the unknown timing we often did "3 drop" sort envelopes to ensure we captured the cell. In other words, each time we wanted to sort we would sort not only the drop predicted to contain the wanted cell but also the drop preceding it and following it. In current sorters, the electronics are fast enough that we usually only sort the one drop predicted to contain the desired cell. However, in some cases, e.g. sorting very rare cells at high speed, we can revert to 2 or 3 drop sorting to ensure we capture the wanted rare cell. We can also do a hybrid type of sorting (termed 1-2 or recovery) where the sorter, based on predicted cell position within the drop will sort only the one drop or the drop preceding or following the drop depending on which side of the primary drop the cell is predicted to be in.

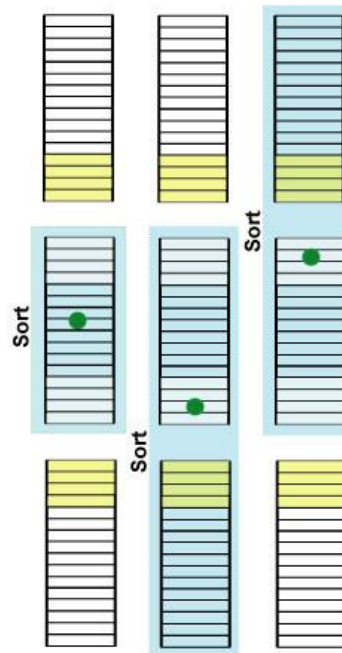


A purity sort mode is designed to produce the highest purity. As can be appreciated, however, this will also result in the lowest yield of the desired cells. In this mode we sort the drop predicted to contain the desired cell only if for some distance on either side of the drop (determined by the operator) we predict that there will not be an unwanted cell. On the MoFlo we cannot do this but the slower electronics actually partially produces this effect. On the MoFlo XDP we can select how far on each side we want the sorter to look for unwanted cells - the distance/time is expressed in a fraction of a drop. We can select anywhere from 0 to 1,000 units of a drop (i.e. 1,000 units is a full drop). Typically we do not do more than 0.25 drops but can lower this. The Reflection is also capable of selecting the minimum separation in time before and after an event. Purity mode is diagrammed in the figure to the left. For simplicity each drop is shown divided only into 16ths. The yellow regions indicate that we have selected a 1/4 (i.e. 4/16ths) drop region on each side of the expected drop for looking for unwanted cells. Obviously if we increase the size of the inspection window on each side of the sort drop we will decrease our yield - more wanted cell sorts will be aborted. If we decrease the size of the inspection window we will increase yield but may decrease purity. Also it should be obvious that as we increase the ratio of cell input rate to drop frequency we will increase the sort aborts (see discussion and table below for cell coincidence rates). The abort rate will also be higher when sorting rare cells because nearly any coincident cell will be an unwanted cell so, in the figure to the left the condition demonstrated third from left will be very rare. In the world of sorting, as with all purifications, it is always a compromise between speed, yield, and purity.



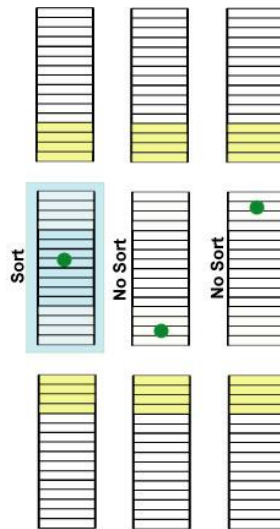
An enrich sort mode is designed to recover cells without regard to contaminating cells. This mode does not look for unwanted contaminant cells in the sort drop. This is typically used when sorting for rare cells at very high speed where the objective is to capture all wanted cells at the cost of having some contaminant unwanted cells. It may also be used as a high speed enrichment with the intent to follow-up with a purity sort. In the figure above, an enrich mode (single drop) would sort all of the middle drops including the 2 examples on the right. Sort envelopes of 1-2, 2 and 3 drops can also be performed.

Yield (recovery) modes are designed to emphasize capturing of cells. Yield sort modes may use either 2 drop sorting or 3 drop sorting but with improved electronics we usually use a mode called 1-2.



The 1-2 mode is shown in the Figure to the left - drops to be sorted are highlighted in blue. In this mode the sorter looks at the predicted position of the cell to be sorted within its drop. If the cell will be near the center of the drop then only the one drop will be sorted (left set of 3 drops). If the cell is predicted to be near the

trailing edge of the drop then the predicted drop and the trailing drop will both be sorted thus increasing the probability of capturing the wanted cell (right set of drops). If the cell is near the leading edge of the predicted drop then the predicted drop and the leading drop will be sorted (middle set of drops). When purity mode is also on with this mode then yield may decrease somewhat since we are looking at a larger drop window for any unwanted cells (only wanted cells are shown in the figure).



If we need to be particularly accurate in sorting a cell, i.e. ensuring that when we sort a drop we have the highest probability of catching the cell and only that one cell, we can do what is called a single sort mode. In this mode we typically sort the drop only when the cell is predicted to be in the center one half of the drop (the Reflection can set the criteria for the center) (see figure to the lower right). If the cell is outside this area, e.g. in the leading 0.25 drop or trailing 0.25 drop we do not sort the drop. In this mode we also usually perform a purity sort but in this case we do not sort the drop if it contains any other cell - wanted or not wanted. We use this mode for example when we want to place a specific number of cells (e.g. one) in a tissue culture multi-well plate well, i.e cloning. The abort rate is pretty high but is not usually a limiting factor except when sorting very rare cells.

Purity and yield sort modes may be combined. In this case the purity will be maintained but yield somewhat compromised due to the slightly higher sort aborts.

Sorting statistics.

As we have seen, cells go through a flow cytometer randomly relative to their arrival at the interrogation laser and at the point where drops break-off from the stream and are charged for sorting. 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. The statistics of the occurrence of events relative to a fixed point in space or time are described by the Poisson statistic. As we describe above, we will have made decisions about how many drops to include in each sort event and how much of neighboring drops we want to have free of cells so we may get the purity or yield we desire.

Three types 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 are identified by the analysis component but too close together to resolve - in the modern (so-called digital) instruments (Aria, MoFlo XDP, Reflection, Influx) this is removed as faster electronics allow individual analysis windows for each cell)
- 3) coincident particles are detected but are too close together to be separated at the sorting stage. Thus, all coincidence in sorting in modern sorters (when cells are resolved by the analysis system as individual cells) comes from the type 3 coincidence.

The type 3 coincidence, and thus the sort rate, can be calculated from the Poisson statistic (see Pinkel and Stovel (Chapter 3) in Flow Cytometry: Instrumentation and Data Analysis, ed. by van Dilla, Dean, Laerum, and Melamed 1985). The formula for the calculation of best sort rate is: $R_s = \epsilon \mu e^{-(1-\epsilon)\mu T n}$ where R_s is the sort rate, ϵ is the fraction of cells to be sorted (i.e. wanted), μ is the input cell frequency (# cells/ μ sec), T is the droplet period (1sec/drop frequency per sec) and n is the number of droplets deflected per sort event. We have prepared an Excel spreadsheet for calculating sort rates that you may download and use (sort calculator). 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 usually 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. Real cells usually have some degree of frank 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.

Note that the calculated sort rate, % sorted, and sort efficiency indicate best possible result. Actual sort rate, and efficiency may decrease primarily based on cell quality especially factors which affect the random delivery of cells to the laser. Also note that the calculations are based on 1 drop sorting. When using other sort modes such as 2 one must input the n value to match the number of drops deflected per sort event. The recovery mode where we sometimes sort 1 drop or 2 is more difficult. For an approximation use 1.5 - this assume 50% of the time 1 drop is sorted and 50% of the time 2 drops are sorted. This is also based on the expectation that the 1 drop sorting is used when the cell is the middle half of the drop. When using instruments e.g. the Reflection where this value can be set that the 1.5 value will be adjusted to reflect the proportion of the drop where 1 drop sorting occurs.