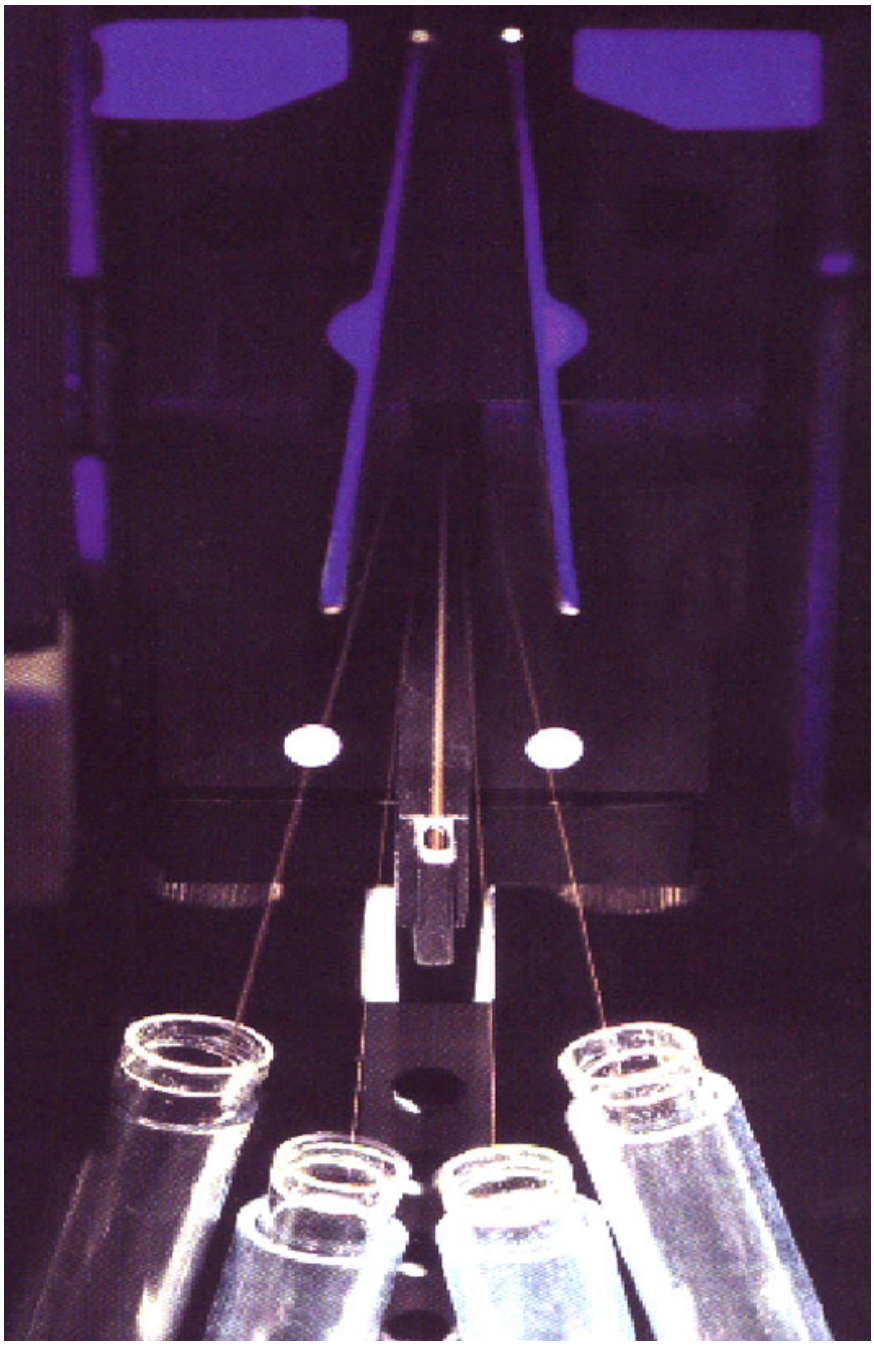
Flow Cytometric Cell Sorting: A Basic Guide

Graham Leslie, Ph.D.



Institute of Medical Biology, University of Southern Denmark

Flow Cytometric Cell Sorting: a Basic Guide

Introduction

Flow cytometric cell sorting (FCCS) is an exceedingly powerful technique for the purifying cell subpopulations from complex cell mixtures. Under optimal conditions, for example, cells occurring at a frequency of 2 per 10,000 can be purified to over 60 % (a 3000-fold purification) in a single sorting step. In this sense, FCCS can be regarded as the cellular equivalent of affinity chromatography. FCCS is, however, a somewhat complex process requiring careful planning to ensure maximum effectiveness.

The purpose of this pamphlet is:

- to provide guidelines for labs interested obtaining pure cell populations (especially ”rare event” cells) with respect to the laying of sort strategies and preparing optimal cell preparations for sorting.
- to orientate the potential users about the realistic goals (in terms of yield, purity and viability) that can be achieved by FCCS, and
- to outline some of the potential pitfalls of this technique.

Flow cytometric cell-sorting: the principle.

Cells are sorted as the “inhabitants” of a stable cascade of droplets generated by forcing a liquid stream through a vibrating nozzle (see Fig 1) The cells are screened on the basis of signals derived by illuminating the stream (just below the nozzle tip) with up to 3 lasers: blue, red and UV.

Following detection of a cell with the desired properties for sorting, an electric pulse is applied to the stream, just prior to the estimated break-off of the cell-containing droplet from the stream (i.e. a few microseconds after detection).

The charged droplet is deflected from the droplet cascade by passage between charged plates and collected in an appropriate receptacle (tube, 96-well plate etc.)

Anything from 1 to 4 different cell types can be sorted simultaneously, depending on the polarity and size of charge applied.

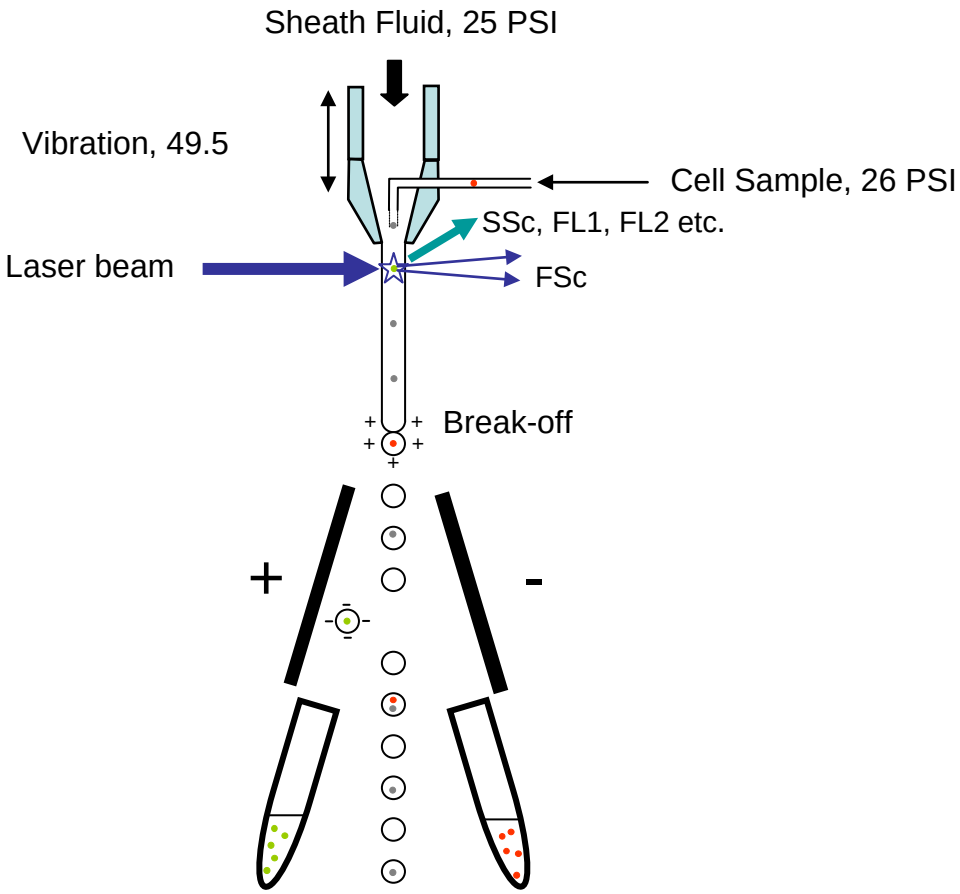


Figure 1. A schematic representation of the sorting process. A cascade of droplets is generated by forcing the "Sheath Fluid" out of a nozzle vibrating at a frequency that provides droplets of uniform size (the resonant frequency). Cells from the sample are injected into the liquid stream by applying a slight excess pressure to the sample tube. The cells are analysed in the continuous liquid stream which emerges from the nozzle. Cells conforming to the sort criteria (on the basis of up to 8 parameters) trigger a brief charging of the liquid stream, just prior the point where a droplet containing the cell is about to break off from the stream. The charged droplet is then deflected from the droplet cascade upon passage between highly charged plates and is collected. In a "purity sort" the presence of an undesirable cell in the same droplet as the candidate cell results in an abort (i.e. cancelling of the charge instruction). In this example, a two-way sort is being performed to isolate "green" and "red" cells from a mixed (red, green, black) population.

Basic considerations

The upper limit for the sorting rate is defined by the number of droplets formed per second. This is determined by the resonant frequency of vibration for the liquid stream (i.e. the frequency at which uniform, stable droplets are formed), which, in turn, is dependent on both the size and velocity of the stream. The size is determined by the nozzle diameter (usually 70 µm), while the velocity is proportional to the hydrostatic pressure of the liquid entering the nozzle. The smaller the nozzle and the higher the pressure, the greater the drop rate.

The actual sorting rate is determined by the number of cells injected into the core of the stream from the sample tube – and thereby the frequency of cells per droplet. This is determined by the excess pressure applied to the airspace above the cell sample.

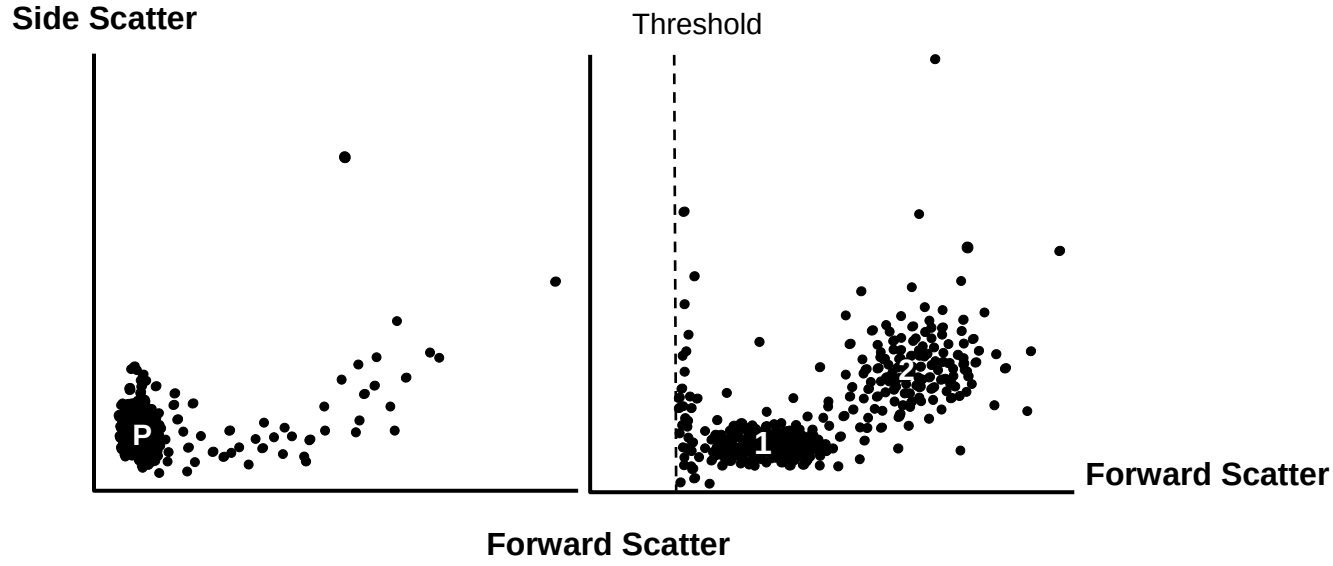


Figure 2. The effect of setting a threshold. Small particles (P) in the supporting buffer for the cell sample will also be registered by the cell sorter and may far exceed the number of cells in the preparation. The left panel shows these events registered in the form of an FSc v. SSc ("size" v. "granularity") dot plot, where the micro-events predominate. Setting a threshold excludes the low-intensity signals derived from these particles (see right panel). Two cell populations (1 and 2) are distinguishable after setting the threshold.

The parameters measured by laser illumination of the cells are the scattering of light along, and at a right angle to, the axis of the laser beam ("forward" and "side" scatter, FSc and SSc) and the presence of up to 6 fluorescent markers.

Background events, in all parameters, are also registered. In the case of FSc and SSc, which measure the physical properties of size and granularity of the cells, low intensity signals (arising from micro-particles in the cell suspension) may be predominant (Fig. 2). Such background events can be excluded by setting a detection threshold on one or more parameters. One should be aware, though, that these events will no longer be seen in the course of a sort, and thus, will remain as contaminants of the sorted cells.

Spurious high-intensity signals ("space dust") may arise in all parameters. Although they occur at a very low frequency, they can lead to sorting deficiencies, especially in relation to "rare event" sorting. However, they are usually "one-dimensional" – i.e. they arise in only one parameter at a time. Thus sorting on the basis of several parameters markedly reduces this interference

Important Factors for Successful Cell Sorting

Quality of the cell preparation

It is important to ensure that the cell sample is as free as possible of cell- and tissue debris, cell aggregates and erythrocytes (E). The presence of tissue debris and/or cell aggregates lead to blockage of the nozzle and thereby interruption of the sort, while E and cell debris will often persist as contaminants of the sorted cells. See "Invisible impurities" below.

It is desirable that the viability of the cell preparation for sorting is as high as possible. While strategies can be applied for the exclusion of dead cells from the sort, their presence compromises sort efficiency

It is essential that the cells are prepared under sterile conditions, both for your own sake and that of subsequent users. Every effort is made to ensure that the apparatus is

kept sterile and your cooperation is required to keep it that way. Colonisation of the fluidics, should it occur, is virtually impossible to eradicate by any method other than replacing all the tubing in the flow system.

Cell density

Cell density is the most important factor (after drop frequency) in determining sort rate.

Sort rate = drop frequency x frequency of cell-containing drops.

For example, with a drop frequency of 50,000/sec. and a cell frequency = 1 cell per 5 drops (which is an optimal ratio), the sort rate will be 10,000 cells/sec.

The *maximal* cell frequency is determined by the cell density in the sample (though it can be modified by varying the proportion of the fluid stream derived from the cell sample – see above).

For *low speed sorting* (up to 5000 cells per second), ca. 3 x 10^6 cells per ml are required.

High speed sorting (ca. 20000 cells per second) requires 1 to 2.5 x 10^7 cells per ml.

As a general rule, the nozzle size should be ca. 3 times the diameter of the sorted cells and the proportion of liquid in the stream, which is derived from the cell sample, should be about ½-2/3 of the total. Too high a cell density will result a high frequency of droplets containing more than one cell (resulting in either poor purity or poor recovery - depending on the sort strategy applied) while too low a density will result in a slow, long sort with corresponding loss of viability.

It is *essential* that the cells are adjusted accurately to the desired density just prior to sorting!

Sorting rate v. viability

The cells are subjected to a hydraulic pressure of anything from 12 to 50 psi, which is suddenly released upon emergence of the stream from the nozzle. Generally speaking, small cells (e.g. lymphocytes) are well equipped to withstand this shock, while larger cells (cell lines, tissue cells, cultured cells) may be more vulnerable. Dendritic cells, for example, do not generally tolerate pressures greater than 15 PSI. For each new cell type, it is highly recommended to perform sorting at various pressures in order to optimise the balance between sorting rate and viability.

Purity versus yield

Sorting can be performed in various modes, ranging from the highly rigorous (rejection of positive cell in a drop if there is slightest chance that the drop in question contains a contaminating cell) to the highly permissive (e.g. collecting two drops if there is chance that positive cell finds itself in a neighbouring drop at the point of break-off).

High purity requires rigorous selection, whilst the yield is influenced both by the degree of permissiveness and, more importantly, by the cell frequency per drop e.g. at a mean frequency of one cell per drop, the likelihood that a drop will contain 2 cells will be 33 %, whereas at 1 cell per 5 drops, the likelihood is reduced to ca 3%. Thus, in a high purity sort, the maximal yield is increased from 67 to 97 %.

NB. If the cells are sufficiently robust, a double sort can be performed, with a first round on the basis of yield and the second on the basis of purity. However, it is advisable at all times to consider other procedures (e.g. magnetic bead separation) for enrichment of the desired population, preferably by removal of contaminating cells.

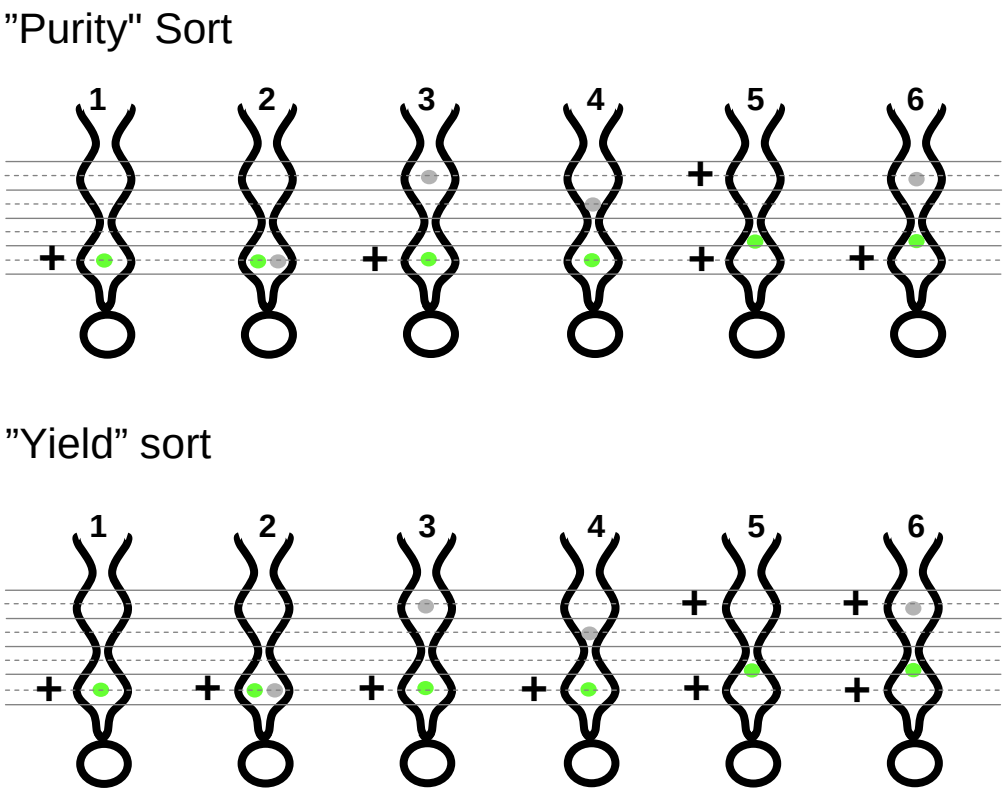


Figure 3. Cell Selection. The flow cytometric data is logged in such a way that each potential drop is recorded as a series of segments (slices). Thus it is possible for the apparatus to discriminate between cells that will come to lie in the middle of a drop (when it breaks off from the liquid stream) and cells that lie close to the forward or rear neck region between the forming drops. Cells lying in the neck regions may end up in either leading or following drops and this uncertainty has to be taken into account when designing a selection strategy. If high purity is desired, uncertainty that might result in contamination of the sorted cells will result in rejection i.e. failure to charge the drop(s) containing a candidate cell (see “Purity”, example 4). If the strategy is to collect as many cells as possible, then charges will be applied to all drops that potentially contain the candidate cells, irrespective of contamination. Horizontal lines: the measurement intervals (32 in reality). Solid lines: the applied discrimination boundaries. + sign: charging of the drop.

“Invisible” impurities

Events, which are excluded by setting a detection threshold (see above) are not rejected under the sort criteria, and will thus be present in the sorted drops. It is therefore of great importance to ensure that cell debris, the size of which may put it under the detection threshold, is removed from the cell preparation *before* sorting, if it is deemed to be threat to the subsequent viability of the cells. You cannot sort your way out of this problem.

Sorting strategies

The effectiveness of a sort is optimised by

- Using as many parameters as possible to identify the cells for selection (see Fig. 4)
- Inclusion of negative criteria i.e. positively identifying a contaminating cell population with an appropriate marker (X) and sorting on the basis “Not-X”.

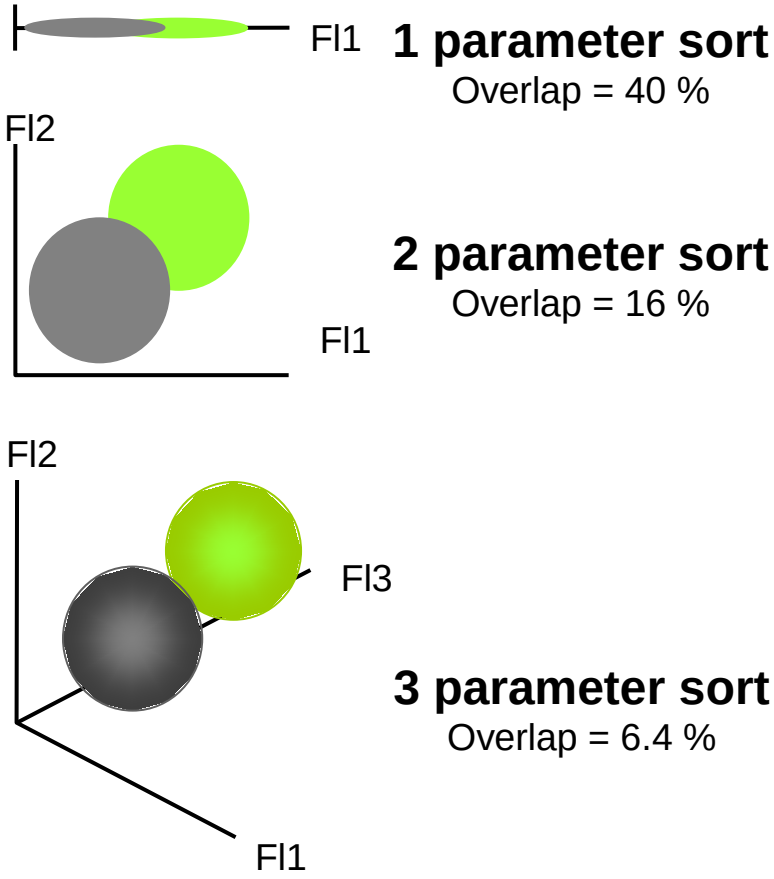


Figure 4 The benefits of multiparameter sorting. In this hypothetical example, the desired cell population (green) expresses three markers at such low intensity that the spread of the fluorescence* seen with each marker overlaps by 40 % the fluorescence signal from the negative cell population (grey). Selecting on the basis of any single marker would thus result in a maximum yield (or purity) of only 60 %. However, if the 3 markers are expressed independently, then discrimination of the two cell populations (and thereby recovery) can be greatly enhanced by including the second and third markers as criteria for the sort.
*Even cells within a cell-line display heterogeneity with regard to the amount of any given marker they express on their surface.

- Choosing appropriate fluorochromes. The different fluorescent dyes employed for sorting vary in intensity. Since it is desirable that the fluorescent signals from all cell markers are more or less of the same intensity, it is advisable that you select your fluorochromes on the basis of marker expression on the cells. For high-density markers, use a weak fluorochrome, such as FITC or APC-Cy7, reserving the strongly fluorescent fluorochromes, such as APC, PE-Cy7 or PE, for the low-density markers. It is a good idea to carry out a preliminary evaluation of the labelling strategy on an analytical flowcytometer, if there is one available locally. However, it should be borne in mind that the analytical machines, which make there measurements on cells passing through a cuvette, are more sensitive than the sorter. Thus, markers that register weakly on an analytical flow cytometer may not be suitable for cell sorting.

Useful fluorochromes:

Excitement at 488 nm (blue laser):
FITC, PE, PE-TX red, PE-Cy5, PE-Cy7, PerCP-Cy5.5

Excitement at 633 nm (red laser):
ASPC and APC-Cy7

UV excitement:
Hoechst 33258 (viability),
Hoechst 33342 (side population sorting),
Indo-1 (Ca^{2+}-flux sorting)

NB. PerCP is an undesirable fluorochrome in that it bleaches too quickly.

Practicalities

The time factor

Cell sorting, even at a rate of 30-40,000 cells per second, is a time consuming process. The day's work in performing a sort consists of labelling the cell preparation, optimising the flow cytometer settings for the cells in question, running the sort and, finally, harvesting the cells and setting them up in culture, not to mention cleaning and shutting down the sorter. Therefore, in addition to the actual time required for the sorting, you should allow about 3 working hours (ca. 2 before and 1 after the sort) to perform all the peripheral activities.

Theoretical run time (sec) =

$$\frac{100 \times \text{Number of cells to be sorted}}{\text{Sort rate (cells/sec)} \times \%\ \text{pos. cells in sample}}$$

The actual sort time can be estimated in advance. Thus, sorting 1million cells, comprising 0.2 % of the starting population, at a sort rate of 30,000 events/second will take 16,667 secs. (i.e. 4h 38 min). However, to ensure recovery of 1 million viable cells, one should reckon on sorting up to 1.6 times as many (i.e. a 7½ h run time). In addition, one should also allow for the possibility of hold-ups arising, for example, from blockage of the nozzle. All in all, a long day!

It is, therefore, important that you take these factors into consideration, in designing your sort, and restrict yourself to the number of cells you actually need, rather than the number "it would be nice to have".

Recovery – the harsh reality

"You always get less back than you expect" – unless you really *are* a pessimist!

*Sorting a 1 % population in a cell sample of 10^8 cells does **not** result in the recovery of a million cells at 100 % (or even 95 %) purity.*

The inevitable losses lie in the following categories:

• During labelling – reckon on 10 % loss per washing step

• Sorting aborts i.e. drops containing the desired cell and 1 undesirable cell). Allow up to 20 % loss

• In the recovery tube (damaged or adherent cells). Allow ca. 30 % loss

• During subsequent washing. See *labelling.*

Labelling may involve up to 5 washes, depending on the complexity of the selection strategy, so an overall loss factor of up to 3-fold is not unrealistic.

It is highly advisable to count the cells just before sorting (to correct for losses during labelling). Even so a recovery of more than 60 % of the theoretical yield should not be expected.

*Sorting a 1 % population in a cell sample of 10^8 cells **can** result in the recovery of 3-500,000 cells, of the desired type, if all goes well.*

Two pearls of wisdom

Howard Shapiro, the doyen of cell sorting, is the originator the following pearls of wisdom:

"a 51µm particle blocks a 50µm nozzle"
"garbage in – garbage out"

The essence of flow cytometric cell-sorting!

Further reading

Flow Cytometry: A practical approach (3rd edition) Ed. M. G. Ormerod, Oxford University Press. *Excellent coverage of all aspects of flow cytometry, both for the beginner and the experienced user.*

Practical Flow Cytometry (4th edition), Howard Shapiro, Wiley Publishers. *The cell sorters's bible.*