Materials and Methods

The Use of Flow Cytometry to Sort Cells or Particles

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Introduction to cell sorting

Flow cytometry is a technique in which a sample (cells, chromosomes, bacteria, micro-organisms) is suspended in a suitable fluid (saline, tissue culture medium etc.) and passed through the path of a laser or focused beam from an arc lamp as a very narrow stream or jet. The rate of flow of a particle is uniform, and can be controlled by adjusting sample and fluid pressures. The light is scattered as it hits or passes through each particle, and if any fluorescent dyes are present they are induced to fluoresce at their characteristic wavelength.

Using suitable photosensitive detectors (photomultiplier tubes), the scattered and fluorescent light can be detected and amplified and, after signal processing, a data set representing each measured parameter can be assigned to individual particles at precise moments in time. Tens of thousands of individual particles in a heterogeneous sample can therefore be measured and, with the use of software packages, data clusters denoting subpopulations within the sample can be identified.

It is these discrete populations that can then be sorted from the rest by virtue of the jet-in-air properties of the sample stream. A solid stream of fluid can be broken into droplets by vibrating it along its length. If the stream is momentarily charged with a positive or negative charge, the last drop to form at the end of the sample stream will hold that charge because it is insulated by air. Charged drops in the sample stream then freefall past two charged deflection plates held at a constant potential of about 3500 volts. Positively charged drops will therefore be attracted towards (but not on to) the negative potential plate, and vice versa. Thus the single stream of drops is teased out to form two additional side streams, one to the left, one to the right, which then fall into the collection tubes. This is illustrated in Figure 1.

Typically a stream 70μm in diameter can be reduced to droplets by a piezoelectric quartz crystal vibrating at a frequency of 30,000/second, and if the cell sample is flowing at a rate of 3,000 cells/second past the light source, then one in ten droplets will in theory encapsulate a cell. As droplets form a short distance below the point of analysis on the stream, the cyto-meter has a few microseconds in which to analyse the sample. The cytometer has to consider:

- is this a whole cell or a particle of debris?
- is the cell labelled with the desired marker?
- is the identified cell in a sort region on the computer display?
- do we sort the cell to the left or right collection tube?
- can the sort decision be made without compromising sort decisions before/after this event?

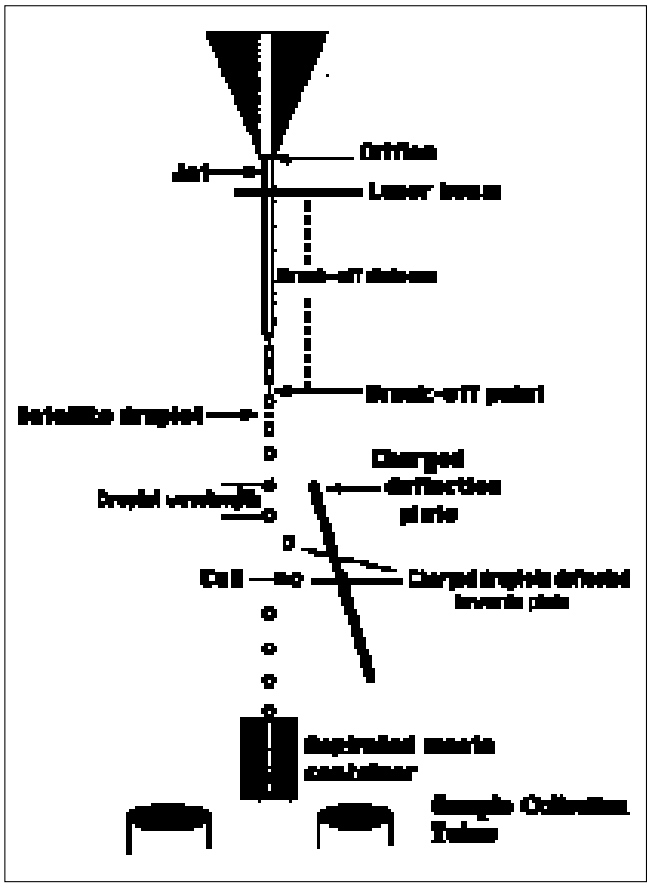

Fig. 1. Setup of a typical flow cytometer

These are all factors that the cytometer operator can influence and therefore can affect the efficiency and quality of the sort. They are also common to all cytometers capable of sorting, including a recently evolved 'bench-top sorter', which moves away from the concept of jet-in-air droplet sorting to a system in which cells of interest are 'caught' immediately after analysis in a flow cell, using what may best be described as a 'scoop' attached to an electromagnetic device capable of oscillating 300 times per second. Actual sort rates are therefore typically about half this rate. However, all sort decision events are the same as those required for the classic cell sorter.

Sample preparation

In general, samples required for sorting need to be freshly prepared, handled with care so as to minimise damage, and include at least one parameter on which to make the sort selection, i.e. a specific light scattering property or specific staining pattern with a fluorescent antibody or other fluorescent label.

Samples required for future culturing should be handled using sterile procedures, and samples required for RNA extraction handled so as to minimise contamination. In addition, all samples should be considered to be a biohazard and appropriate precautions taken, especially when the sort is under way and potentially dangerous aerosols can be produced.

Cytometer preparation

Two aspects of cytometer preparation should be considered: one to ensure the sterility of the sample to be sorted, and the resulting sort populations; the other to ensure the safety of the operator.

Both aspects require suitable cleaning procedures before and after the sort.This usually includes using a new sterile sheath fluid filter before each sort, and using sterile sheath fluid; swabbing the whole sort chamber with 70% alcohol before the deflection plates are activated; running a 'sample' of 70% alcohol, then sterile medium, before the sort sample is introduced; and ensuring that waste sample is removed using an efficient vacuum line to minimise aerosol formation which might contaminate the pure separated samples in the collection tubes.

Both aspects of preparation also require the minimum of disturbance during a sort. Disturbance is usually associated with opening the sort chamber door to remove full tubes or to clean a blocked nozzle. The latter should be prevented by filtering the sample, mixing intermittently, resuspending in a suitable medium, using good quality (viable) cells for the sort, and if necessary using serum and/or DNAase to minimise cell/cell adhesion, which results in clumping which may block the nozzle.

Sort rates

Table 1 illustrates typical sort times based on a flow rate of 3000 cells/second. A range of sort populations of interest from 0.1% to 20% of the total sample are shown, and several typical yields are included to illustrate how rapidly sort times can build up when large numbers of rare cells are required. Clearly such conditions would normally require pre-enrichment, either by rapid, low purity cell sorting, or by the use of other enrichment procedures such as magnetic bead separation.

Viabilities and yields

It is generally considered to be the case that the physical aspects of the cell sort do not reduce viability for most cell types. Neutrophils, megakaryocytes and plant protoplasts may be the exceptions; sorted cells should be as viable as the original sample. Sample preparation is therefore the critical stage dictating the success or failure of the cell sort.

Sort yields are dependent on two factors. The first is the ability of the cytometer to clearly distinguish the cell of interest from all others and to establish perfect sort conditions to enable the charged droplet to reach the collection tube. Sort events will be aborted if a cell is too close to its neighbour, and if a full charge cannot be applied to the drop. Thus the critical factors influencing abort rates are the speed of sample flow and the rarity of the population of interest.

The second factor affecting viability is the environment in which the cell finds itself. Cells may clump together and they may also adhere to the walls of tubes and thus be unrecoverable. This can be reduced by rinsing tubes with medium/serum which should minimise both biological and electrostatic cell adhesion. The sample should be filtered before sorting to remove cell clumps, and resuspended in medium containing serum to prevent the formation of further clumps.

Sorted cells should be harvested at regular intervals to remove excess sheath fluid, which is often only saline and which over prolonged periods may reduce viability. Unless the cells are damaged by low temperatures (e.g. neutrophils, spermatozoa) all samples should be held on ice, and the sort tubes and sample tubes cooled with circulated ice water.

Table 1. Typical sort times.

Number of cells requested	0.1% total	1.0% total	10% total	20% total
1,000	5.5 min	33s	3.3s	1.7s
10,000	55 min	5.5 min	33s	17s
100,000	9h 15 min	55 min	5.5 min	2.8 min
1,000,000	3 days 20h	9h 15 min	55 min	28 min
10,000,000	38 days	3 days 20h	9h 15 min	4h 40 min

Sort recovery

Owing to a slight positive charge on stream illumination lamps, the negatively charged sort droplets in the right-hand stream may be attracted towards the lamps in the sort chamber. Thus one solution would be to switch off the lamps. Alternatively, it is generally recommended that the sort population with the lowest frequency (i.e. rare cells) should be sorted to the left; under these conditions the sort drop is given a positive charge and is not influenced by the lamp as it free-falls past.

If static charge, resulting in the sight of sorted drops 'bouncing out' of the collection tube, seems to be a problem, an earth (ground) wire should be fitted to the sides of the collection tubes and connected to a suitable point on the cytometer chassis or optical bench.

Sort containers

Whilst the conventional sort container is a sterile 5 or 10ml tube, sorters may be equipped with single cell deposition units that enable the sorting of individual cells into individual wells in 6-, 12-, 24- and 96-well tissue culture plates. In addition, particles may be sorted on to microscope slides or coverslips, into Petri dishes, ampoules, bijou bottles etc. I have sorted on to coated electron microscope grids, although care has to be taken to sort the correct number to cover the 'gaps' between the grid, as the charged droplets have a tendency to be attracted towards the metallic component. With minimal modification (and some Blu-Tack), sorts can be made into any suitable containers including cytocentrifuge holders, Millipore filter holders and so on.

It is clear that many advances have been made and many new applications found for the technique of flow cytometry. Some of the latest include:
- analysis of intracellular cytokines
- analysis of intracellular antigens
- antigen quantification
- measurement of intracellular enzymes.

Cell sorting is a powerful feature of flow cytometry, and scope for its use is as wide as your imagination. Who knows what the future has in store?

Acknowledgements

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