B.- Flow cytometric cell sorting

Flow cytometry sorters allow physical separation of sub-populations of cells of interest from a heterogeneous population, with a high degree of purity, for further analysis. Two kinds of sorting mechanisms exist:

1.- mechanical sorting : within a flow cell

Some flow cytometers, such as the FACSCalibur, use a mechanical device called the « catcher tube » to sort the cells of interest. The catcher tube is located in the upper portion of the flow cell and moves into the stream to collect the cells (fig. 1). When cells pass through the laser beam the system determines if each cell belongs to the selected population defined by boundaries in the cytogram. If the cell is identified as a cell of interest, it is captured by the catcher tube and collected into a tube or into a concentration module, otherwise it is dispatched to the waste tank. The operator can choose the purity level of the sort among three levels (three sort modes).

2. electrostatic sorting : « stream in air »

Most of the high speed cell sorters use the electrostatic deflection of droplets method. In this case there is no flow cell but the stream is focused in a vibrating nozzle and exits in a jet which is broken into regularly spaced droplets (fig. 2). The droplets containing a cell of interest are charged electrically (positively or negatively). When a charge droplet passes through a high voltage electrostatic field, between the deflection plates, it is deflected and collected into the corresponding collection tube. The deflection of the droplet is towards the oppositely charged plate, so that this droplet is separated from uncharged and oppositely charged droplets. In this case it is possible to sort two different populations of the same sample. Generally the sorted cells are collected in a tube but they can also be collected on slides or in multi-well plates. For precise sorting it is very important to adjust several parameters, including:

- the nozzle vibration conditioned by the ddf (drop drive frequency which is the number of drops formed per second) and its amplitude level,

Figure 1: (Left) catcher tube in sheath stream; (right) catcher tube in sample stream (BD Biosciences)
- the particle rate, i.e. the speed which influences the distance between each cell,
- the dead time: time taken by the instrument to measure a particle’s signal and reset to measure the next particle (i.e. time necessary to analyze one particle),
- the drop delay: distance between the laser beam interception of the cell and the break-off point, the point where the stream breaks into droplets…

The operator can choose the purity level of the sort among four levels (four sort modes). The FACS Vantage SE can sort up to 10 000 cells/second when it is equipped with a turbo sort option and the appropriate nozzle.

Note: The orifice diameter of the nozzle tip used for sorting should be seven to ten times the diameter of the cells to be sorted.

The main drawbacks of the mechanical sorting is the possibility to sort only one population of cells at a slow speed of sorting. Nevertheless there is no aerosol, so that it is safe to sort samples which have been treated with toxic substances such as radioactive compounds.

The advantage of the electrostatic sorting is the possibility to sort two sub-populations of cells at a high speed. However it generates aerosols so it is not appropriate to sort samples which have been treated with toxic substances. The high pressure generated in electrostatic sorting can also damage the sorted cells.

COMMENETS

a.- Sheath:

FACSCalibur: generally the sheath has the same salinity of the analyzed sample. For fresh water samples, PBS or milliQ distilled deionised water is used, but for seawater samples, 0.2µm filtered seawater is used as the sheath fluid.
FACSVantage SE: for the stream in air system sorting systems, sorting requires a conductive medium that is chosen according to the sample. The sheath fluid consists of sterile 0.2µm filtered PBS for fresh water samples and sterile 0.2µm filtered seawater for marine samples.

Caution: Several manufacturers sell commercial sheath fluids, but they contain detergent which could affect cell viability.

b.- Maintenance:

Weekly and monthly maintenance must be performed according to manufacturer’s recommendations in order to maintain system performance and to ensure the long-term functioning of the system. For the stream in air sorter, it is important to clean the nozzle tip and the deflection plates by sonication in deionised water after each use.

Note: Special cleaning is recommended for the FACSCalibur after each use as residues of some dyes can stick to the flow cell. This can cause significant background in subsequent analyses or the appearance of a parasite population. So we recommend after each use one « prime » of the flow cell and a subsequent run of FACSClean for at least one minute in high speed, one more « prime » and a run of at least one minute of FACSRinse.

c.- Cleaning the flow cytometer before each sort

The cytometer is cleaned before each sort in the same way as the weekly maintenance. The saline filter is disconnected, the sheath tank is filled up with FACSClean (equivalent of bleach) and a tube of FACSClean is run for 30 minutes. Then the tank is filled with FACSRinse and a tube of FACSRinse is run for 30 minutes. The sheath tank is then filled up with the appropriate sheath fluid and the flow cytometer is rinsed for 10 minutes. The system is then ready for cell sorting!

Note: The saline filter is kept disconnected during the cell sorting because it could be a source of bacterial contamination (biofilm) if it is changed according to the manufacturer advice every three to six months. The other solution to this problem is to replace the filter just before each cell sorting. But since the sheath fluid is filtered through a 0.2µm sterilisation filter, this is not necessary.

d.- Output verification

FACSCalibur: the output verification is done by sorting 200 fluorescent beads which are harvested on a membrane filter. This membrane is then analyzed by the ChemScan (solid phase cytometer, Chemunex, Ivry-sur-Seine, France). The number of beads counted with the ChemScan allows the calculation of the output. This is done in triplicate.

FACSVantage SE: The output verification is evaluated by re-analyzing the whole volume sorted. This is done arbitrarily with 5 x 10⁵ sorted cells (but it can be done with fewer or more cells). The tube in which the cells are harvested is vortexed, then centrifuged (1 minute at 3000rpm), vortexed again and re-analyzed by the flow cytometer in acquisition mode but it is necessary to select the analysis depending on the cell number (and not the time). This number must be very high (much higher than the number of cells that will be analyzed). When the tube is empty, click on pause then save. The number of re-analyzed cells then allows the calculation of the sorting output. This is done in triplicate.

e.- Purity control

The purity of the sort depends on the sort mode selected. The purity control is done in the same way as the output verification: the sorted population must be sorted among several populations. The re-analysis of the sorted sample allows one to assess if cells of the other populations are sorted or not (directly on the cytogram if the re-analysis is done with the flow cytometer or on the microscope if the re-analysis is done with the ChemScan).

f.- Sheath control

It is very important to check the purity of the sheath fluid, especially if molecular biology tests are performed on the sorted samples. An aliquot of the sheath is taken at the sort exit. The harvested volume must be the same as the sorted cells sample volume.
g.- Flow rate calibration:

The flow rate calibration is important to quantify the cells per volume unit. Two methods can be used for the calibration of the flow rate of the FACSCalibur:

- Weight differences: three tubes with the same volume of milliQ water as the analyzed samples (generally 1ml) are weighed, then run 5 minutes and weighed again. The weight difference of the samples before and after the run gives the volume aspirated by the cytometer in 5 minutes at the selected speed. It is then possible to calculate the mean of the flow rate per minute.

- Calibrated bead suspension: a suspension of concentrated beads (surfactant-free Fluorescent yellow green CML polystyrene latex - Interfacial dynamics Corp. Portland, Oregon, USA) is prepared at about $10^6$ beads/ml and precisely calibrated with the ChemScan. These beads do not aggregate, and therefore only single beads are counted when the suspension is calibrated by the ChemScan. The bead suspension is sonicated for one minute (in order to resuspend beads stuck to the tube wall) and is then added to each sample to be analyzed at a concentration of 1%. On the cytogram, only the single beads are gated, then the number of beads analyzed and the analyzed time allow the calculation of the flow rate.

- Volume differences: this calibration is done in the same way as the weight differences, in triplicates. The volume is measured before and after the run of five minutes with a micropipette (the best ones are the Gilson P1000). However, these measurements are not as precise as those done using weight differences.

Note: the outer sleeve of the sample injection port (SIP) is removed permanently. Otherwise, when the tube support arm is open, the sheath fluid continuously circulates between the sample injection tube and the outer sleeve, in order to rinse the system. In this case it is impossible to control the added volume to the sample to be analyzed when the tube is installed on the SIP. When the outer sleeve is removed it is easy to control the drops of sheath fluid: the tubes are placed quickly on the SIP after a drop fall, just before the following drop appears.

Figure 3: FACSCalibur unit and Sample Injection Port (BD Biosciences)
e. Safety rules:

- Laser: modern cytometers do not allow the user to have direct eye contact with the laser beam, but caution must be taken with the reflection of the beam from jewellery or tools (such as tweezers).

- Optics or electronics (and power supply) and fluids do not like each other!

- Sample hazard: The user must be aware of the risks of the sample and its preparation. Caution must be taken with pathogenic organisms, with toxic fixative solutions, and with the dyes or the probes used to stain the cells.

Two other minor hazards must also be taken into account:

- Hearing hazard: continuous use of the cytometers and the humming of the air-cooled system or the noise caused by the sorting device of several mechanical sorters may cause hearing loss.

- Sight hazard: continuous use of the cytometers and particularly the screen of the computer may cause damage to eyesight.

Figure 4: Picture of the FACS Vantage sorting system