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Fluorescence Activated Cell Sorting (FACS): An Advanced Cell Sorting Technique

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Abstract
Fluorescence-activated cell sorting (FACS) follows the principle of flow cytometry. It is used to separate the molecules or cells of interest from the heterogeneous mixture by selecting the specific cells. The sample is prepared by labelling the cells with fluorescence dye. It is excited by a laser source which results in scattering and fluorescence due to absorbed light. A number of systems have been produced using two or three lasers to excite several dyes. The light scatter and fluorescence detector outputs are amplified and processed for evaluation. The function of fluorescent excitation and detection system is to produce the signals which accurately reflect the amount of each dye associated with the cell. It is applied in the biological research field, i.e. lymphocyte determination, isolation and characterization of infected stem cells, screening of protein binding with various ligands and for the quantification of nucleic acid content in a cell.
Keywords: Fluorescence-activated cell sorting (FACS); signal detection and amplification; proteins; stem cells; nucleic acid.

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INTRODUCTION

Herzenberg and his colleagues invented FACS in 1960 to sort the cells and do flow cytometry. Other contributions were done in 1970 by Becton Dickinson group. They invented machines for the progress of FACS. In early stage two light detectors and one laser beam were used, but in the modern era thirty thousand sorters and analysers are present in the market. Fluorescence-activated cell sorting (FACS) follows the principle of flow cytometry. It can separate the molecules or particles of interest from the heterogeneous mixture by selecting the specific cells. These specific cells get illuminated by a laser light and show the characteristics of our interest. Then these cells get charged in a fluid as single droplets and then exposed to electrical field. Due to the presence of an electrical field, these droplets which have cells of our interest will go into different containers and rest of the debris will separate out (Herzenberg *et al.,* 2002; Herzenberg *et al.,* 1976). The cells that get separated on a decision that how much fluorescent dye is bound to each cell. The current model, FACS-II, detects cells with as few as 3,000 molecules of fluorescein (a common fluorescent dye) on each cell (Bonner *et al.,* 1972). In addition, by means of correlated light-scattering, the device simultaneously measures the size of all cells; hence it can be set to separate those cells that fall within desired ranges of both size and fluorescence. FACS has the ability to recognize 12 dye's fluorescence. The cells can be stained, analysed and sorted simultaneously by FACS (Wang *et al.,* 2005).

Components of FACS

The sample is prepared by labelling the cells with fluorescence dye. Fluorescent and scattered light are produced when a cell contained in the jet passes through a focused laser beam. A number of systems have been produced using two or three lasers to excite several dyes (Mattanovich and Borth, 2006). Use of such systems may require three or four fluorescence detection channels. The jet consists primarily of cell-free sheath fluid. The cell suspension is injected through the inner nozzle of a coaxial nozzle assembly. Cell-free sheath fluid flows around the inner nozzle, accelerating and narrowing the inner stream. The fluids emerge coaxially from the nozzle. The relative flow rates of sheath and sample fluids are determined by the differential pressure established by the relative heights of their respective supply reservoirs, both pressurized from a common compressed air source (Valli *et al.,* 2014). Typically, an average pressure of 0.84 kg/cm^2 (12 lb/in.2), and an excess head of 30 cm for the sheath fluid, produce a jet

velocity of 10 m/sec, a sheath flow rate of 0.02 ml/sec, and a sample flow rate of 0.003 ml/sec (Fu *et al.,* 1999).

The cylindrical liquid stream is illuminated immediately after emergence by an argon laser (other lasers used for excitation may include krypton-ion laser, tunable dye lasers, etc.) which can be tuned to any one of a number of wavelengths between 454 and 514 nm. Output at 488 nm, the most frequently used line, is 300 mW. A combination of spherical and cylindrical lenses focuses the beam to a narrow ellipse at the intersection with the stream axis. The laser beam intersects the stream axis at an angle of 45°, to prevent the direct beam from reaching the detector (Herzenberg *et al.,* 1976). A cell crossing the laser beam on the stream axis is illuminated for several microseconds, during which time it emits a fluorescent pulse. Some of this light is collected by a microscope objective (positioned to minimize the entrance of direct laser light) and directed to the cathode of a photomultiplier tube after passing through the filters. Thus a signal pulse is generated at the photomultiplier output whenever a fluorescent cell crosses the laser beam (Tung *et al.,* 2004). If the signal pulse from a cell is within predetermined amplitude limits, a charging pulse is generated electronically, after a delay corresponding to the transit time of the cell from the laser beam to the point where the drops break off (Parks and Herzenberg, 1984). This charging pulse is applied to an electrode which makes contact with the electrically conducting (isotonic) fluid in the nozzle. The pulse induces a charge on the stream proportional to the applied voltage (Davies, 2007). The drops, which break off from the stream while the pulse is applied, including the drop containing the desired cell, carry a sample of the charge existing at the instant they separate. After being selectively charged in this way the droplet stream passes through a transverse electrostatic field established by two parallel deflection plates having dc potentials of ±1000 V (Dubitzky *et al.,* 2013). Charged drops are deflected transversely by an amount proportional to their charge. After traversing 10 cm of deflecting field, the trajectories of charged and uncharged drops are separated by a few millimetres. They may then be easily collected in separate collecting tubes positioned in the stream path (Nishiya, 1988).

Droplet size and spacing are made precisely uniform, and the point at which drops separate from the parent stream is stabilized by vibrating the nozzle assembly along its axis at a frequency of 40 kHz with a piezoelectric transducer (Amor *et al.,* 2002). The resulting velocity modulation of the stream produces small variations in stream diameter. These are further amplified by surface tension forces to decompose the jet downstream from the observation point into very uniform droplets which form in synchrony with the applied vibration. Modulation of the stream diameter at the observation point modulates both the incident light and the fluorescent signal. To minimize this effect, the vibration amplitude is made as small as possible, consistent with stable and uniform drop formation, and the stream is illuminated soon after it leaves the nozzle (Hulett *et al.,* 1969). Because the droplet geometry is uniform, the charge carried away by each droplet and thus its subsequent deflection is precisely determined by the charging voltage. Each charging pulse must arrive at the appropriate time to separate the droplet containing the selected fluorescent cell (Suzuki *et al.,* 2004).

Experiments have shown that the time delay between observation of a cell and its capture by a separating droplet (typically 150 µsec) is predictable to within one drop period. A charging pulse width of 75 µsec, sufficient to charge three droplets, is used to ensure separation of the wanted cell (Caron, 1998). The deflection pulse generator is retrigger able so that cells following one another at less than 75 µsec intervals will result in the generation of an appropriately longer pulse. Processing to the signal processing electronics, the light scatter and fluorescence detector outputs are amplified and processed for evaluation. These signals are digitized and fed to a computer or the pulse height analyser for storage and analysis (Baret *et al.,* 2009). Cell frequency data in one or two of the scatter/fluorescence measurement dimensions can be displayed on a CRT screen or on a hard copy plotter to assist the investigator in visualizing and interpreting the cell population measurements (Muller and Nebe-von-Caron, 2010). Figure 1 shows the working of electronics of signals. The signal pulses from a photomultiplier tube are amplified to peak amplitudes between 0.1 and 10 V. The amplified signal feeds a "single channel analyser" that generates a trigger for each input pulse between selectable amplitude limits, and a pulse height analyser system. Figure 1 shows the components of FACS.

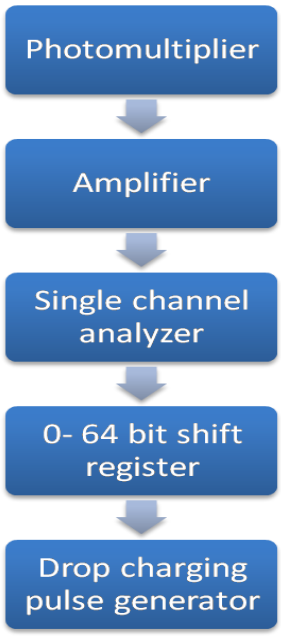

Fig. 1. Components of FACS (Parks and Herzenberg, 1984).

Each trigger from the single channel analyser, delayed by up to 400 µsec in a 0-64 bit shift register clocked at 160 kHz, initiates a drop charging pulse. A 40 kHz synchronizing signal permits on or off transitions of the charging pulse to occur only midway between formations of successive drops, ensuring that each drop that forms during the pulse is charged to the same value (Francisco *et al.,* 1993). Sorting decisions can be made using either analog or digital data. In analog system the cells are sorted on the basis of signals that are given by laser light and the signals of fluorescence dye. These signals then get combined with a shape of a box. The signals whose values are in the limitation of the box will be sorted out and those signals which are out the box range will not be sorted out. The digital sorting is done by a computer program; the selection may be defined on the basis of a more general combination of the signal values (Parks and Herzenberg, 1984).

Operational Concentrations and working of FACS

The cells passing through the machine must retain both viability and function in order to be useful for many biological experiments. It is found desirable to operate with cell and sheath flow suspensions and containers for the emerging streams in an ice bath. Under these conditions, there appears to be little if any damage to the cells from passing through the unit.

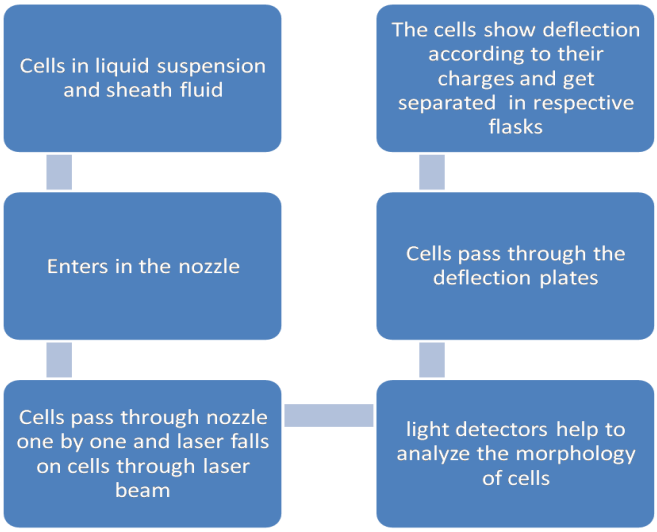

Fig. 2. Working of FACS (Parks and Herzenberg, 1984).

In common operation, we substantially reduce the time required to separate large numbers of cells by using two passes, processing the first time at high rates-several thousand per second and then running the processed fractions through a second time to achieve the necessary purity. Even in this case, there is no indication of cell damage. Typical cell concentrations are in the range from 105 to 107 cells/ml, although up to 109 has been used without undue trouble from blockage (Parks and Herzenberg, 1984). However, since flow rates of the cell suspension are about 3 X I0-3 ml/sec, concentrations much higher than 107 will average over one cell in each drop generated (Figure 2).

Separation efficiency

Unwanted cells from the input mixture divide between the deflected and un-deflected output fractions in the ratio of deflected to un-deflected droplet rates. The required purity of the deflected fraction sets a limit to the rate at which cells can be processed and makes a charging pulse of minimum width desirable (Porter *et al.,* 1993). For example, if fluorescent cells are separated from a mixture at the rate of 2000 cells/sec, 6000 droplets/sec must be charged. Since this is 15% of the 40000 droplets generated per second, the deflected fraction will contain, in addition to the desired cells, 15% of the unwanted (passenger) cells from the input mixture. On the other hand, if only 200 cells/sec is separated, only 1.5% of the passenger cells should be deflected. We are presently incorporating a second signal processing channel which will detect all cells, utilizing their light scattering properties, and which will inhibit drop charging if an unwanted cell would otherwise be deflected along with a fluorescent cell.

Fluorescence Analysis

The function of fluorescent excitation and detection system is to produce the signals which accurately reflect the amount of each dye associated with the cell. For good sensitivity in immunofluorescent measurements we need to maximize the number of photoelectron produced at the photomultiplier tube (PMT) photocthode by fluorescence of our dye and minimize the interference due to light from other sources. The rate of photon emission by a cell is proportional to (1) the amount of dye associated with the cell, (2) the illumination intensity, (3) the extinction coefficient of dye at exciting wavelength, and (4) the quantum efficiency of the dye for fluorescent emission. The conversion of fluorescence emission to photoelectron is governed by (1) light collection efficiency of fluorescence optics, (2) transmission of optical filters for the light emitted by the dye, and (3) quantum efficiency of PTM for producing photoelectrons from filtered florescence. Optical masking and filtering in fluorescence measurements is used to pass as much light as possible from the dye while passing minimum possible light from any other source (Dubitzky *et al.,* 2013).

Flourescence dyes used in FACS

The dyes which are used in FACS are fluorescein, rhodamine, Texas red and Green fluorescent protein (GFP) (Flores *et al.,* 1984).

Monoclonal Antibody

The discovery of hybridomas by Kohler and Milstein made FACS a laboratory and clinical standard. Hybridomas

produce unlimited amounts of distinctive monoclonal antibodies, each of which are highly specific for its target antigens and can readily be coupled to fluorescein, phycobiliproteins, and other fluorochromes (Perez, 2002). The use of monoclonal antibodies as FACS reagents has enabled definition of hundreds of target antigens present on or in cells (Fiering *et al.,* 1991).

The FACS not only uses monoclonal antibodies, but also uses them during hybridoma development to sort and clone individual cells from a hybridoma fusion. Single-cell sorting with the FACS is the most efficient way of cloning cells, especially when they are present at a very low frequency (Weston and Parish, 1990).

Characteristics of FASC

It has some characteristics according to its capabilities:

- It is highly efficient in its action. It has efficient flow cytometer which can quickly detect the hundreds of cells and isolate specific cells, which are necessary for further research (Lanier and Loken, 1984).
- It isolates molecules in highly purified form by the interactions between antigens and antibodies.
- It isolates or separates only specific number of cells or molecules in committed container which are important for research. By this step it concise the number of cells (Schmid *et al.,* 1994).

Parameters that can be detected by FACS

This technique is based on the flow cytometry that's why it has same detectable parameters which also required by flow cytometer.

- It can detect the size and amount of cell content.
- It can also detect the different cell components e.g. cell organelles (Samsel and McCoy, 2016).
- It can examine the different functions of cells, e.g. protein modification, protein expression and localization
- It includes the detection of extra compounds, e.g. transgenic products in-vivo specifically green fluorescent protein or fluorescent markers (Longin *et al.,* 2015).
- It can detect the enzymatic activities in cell.
- Different combinations of the detectable markers such as DNA/surface antigen, etc.

Advance in FACS

This is a modern form of FACS and it has 3 laser sorters which are attached with multiple detectors. This modern form has made it possible for the detection of twelve different fluorescent colours at a time. It also has considerably increased the analysing and sorting speed. It has some advantages.

- Economically favorable because it combines multiple reagents in less apparatus or allows more tests per sample.
- Subset identification can be combined with intracellular metabolic measurements, e.g. calcium, DNA, mitochondrial indices.
- Detailed phenotyping of precious samples with small numbers of cells.
- More reagents per sample reduces the number of separate stains and therefore the amount of sample required (Mathur *et al.,* 2016).

Applications of FACS

The aim of sorting is to separate the interested cell from the sample for further research or clinical use, and if only the special character that is different from other cells can be detected by the flow cytometer the sorter will be able to pick it out. Cell sorting cannot only give you a pure cell population but also can control the population's concise cell number. This character is very important and can enable us to do a lot of things we need. The following are some examples for cell sorting applications:

1. To get pure population of the interested cells for special research or clinical use.

a. Eliminating dead cells for accurate internal staining of cytokines: Cells are incubated with EMA, produced by Molecular Probes and then EMA enters dead cells (membranes not intact) and intercalates into the DNA. Exposure to light from a fluorescent lamp causes covalent linkage of EMA to DNA. EMA fluorescence remains associated with the dead cells then EMA stained cells can be gated out before analysis of the FACS data.

b. Sex control of birth: The yield of flow cytometric sorted X- and Y-chromosome bearing sperm in a given time period is an important factor in the strategies used for fertilization and the production of sex-preselected offspring. With the help of the flow cytometer sperm can be divided into X-and Y chromosome- bearing group, then they can be used for fertilizer to control the sex of offspring.

2. To control the concise number of the interested cells for special research or clinical use. For example: the sorter can easily recognize and drop a single interested cell to a well or tube so that single cell research can be done.

3. Get the rare interested cells from vast back ground cells.

a. Residual tumor cells
c. Neonatal blood
d. Fetal cells in maternal blood

4. FACS is useful in pharmaceutics.

Depending on the client choice, confluence discovery technicians, develop assays for detection of whole blood cells, matrixes in cell structure.

5. Physiological uses:-

Cell practicality is likely the most generally utilized parameter in this regard. An exhaustive investigation of reasonability evaluation by stream cytometry and cell sorting has been portrayed by Nebe-von Caron et al. By triple fluorochrome recoloring utilizing propidium iodide, ethidium bromide and bis-oxonol, it is conceivable to segregate between undamaged, harmed (layer depolarized) and dead cells, which was checked by sorting and plating of the distinctive subpopulations. Comparative methodologies have been taken after for lactic corrosive microscopic organisms ,

encourage hidden the legitimacy of fluorescent reasonability recoloring. Trance like states Riu and Vives-Rego expanded this idea for Paenibacillus polymyxa by including the forward disperse motion into the appraisal, in this manner separating amongst live and dead vegetative cells and in addition reasonable and non-practical endospores.

6. Protein engineering:-

The streamlining of protein structures to enhance particular elements like enzymatic movement, specificity, official to ligands, proclivity, or dependability is a critical errand for the improvement of biotechnological items and procedures. While normal outline of proteins is a testing assignment, screening of irregular libraries has been turned out to be an important option in various cases. A traditional arbitrary screening methodology is phage show of counter acting agent libraries. Phage show can be viewed as the main case of surface show systems, which share the normal rule that a protein which is encoded in the genome of a host cell (or an infection) is shown as a combination protein on the external surface of a similar cell. Therefore, the hereditary data is constantly connected to the individual protein variation in a similar cell. Various utilizations of phage and cell surface show have been distributed. When in doubt, the objective polypeptide is melded to a local surface bound protein, be it on an infection capsid, the cell film or the cell divider.

CONCLUSION

Fluorescence activated cell sorting technique is used to sort the cells by measuring multiple parameters simultaneously from hundreds of cells. Previously two light detectors and one laser beam used but in the modern era thirty thousand sorters and analysers of FACS are present in the market which are more sensitive for detection purposes.

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CONFLICT OF INTEREST

The authors declare that they don't have any conflicts of interest and are also not interested in competing with anyone.

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