Two use cases for Flow Cytometry

Background on Flow Cytometry

In flow cytometry, intact cells and their constituent components are tagged with fluorescently conjugated monoclonal antibodies and/or stained with fluorescent reagents and then analyzed individually. In the instrument, hydrodynamic forces line cells up in single file and the fluorescent molecules in each cell are excited by laser light. The fluorescence emission from each cell is collected by a series of photomultiplier tubes and the subsequent electrical signals (events) are collected and analyzed on a computer that assigns a fluorescence intensity value to each signal. Flow cytometers have become very efficient and can analyze 5-20,000 cells per second and identify rare cells that represent one of every thousand cells in mixed populations. Complex multiparametric data can be collected on many thousands of cells in a matter of minutes. In addition, thousands of fluorescently conjugated antibodies and fluorescent dyes are now commercially available to provide a wide array of cellular measurements including cell phenotype, intracellular cytokine expression, cell cycle status and signal pathway activation. As a result, FCM is widely used clinically including to, for example, measure immune reconstitution following bone marrow transplant and for HIV/AIDS diagnosis and monitoring.

Use Case #1 "Analysis"

Objective: An investigator is trying to isolate hematopoietic stem cells using flow cytometry for further study.

Specimen collection

Peripheral blood specimens are collected from a patient by venepuncture into an anticoagulant (K_2EDTA or K_3EDTA) and a WBC is obtained within 6 hours of the venepuncture. The sample is labelled with the patient's surname and forenames, a unique patient identifier such as the hospital reference number and date of birth as well as the patient location and the date and time of collection. The sample is stored at 4^0C and is processed within 12 hours. The sample may be packaged and transferred, but the specimen integrity must be checked for clot formation, gross haemolysis, or if the specimen was collected more than 12 hours from time of receipt.

Specimen preparation

Peripheral blood specimens are labeled with phycoerythrin (PE)-conjugated CD34[1] antigen and a FITC-conjugated monoclonal antibody to the CD45 antigen. Cells are sampled and detected in a flow cytometer with an argon laser. The antibody combination enables the identification of true $CD34^+$ cells and simultaneously provides a useful indicator for the effectiveness of red cell lyses. To achieve a intra-assay coefficient of variation (CV; expressed as a percentage is 100 times the standard deviation / arithmetic mean) of 10%, a minimum of 100 $CD34^+$ events are collected in data files in Flow Cytometry Standard[2] (FCS) file format.

Flow Cytometer Settings

The daily performance of the flow cytometer is monitored using unstained and fluorescent latex beads. The light scatter and fluorescence peak channel coefficients of variation, light and fluorescence peak channel drift, and instrument sensitivity are all monitored and logged daily along with instrument settings. In addition, a process control is performed weekly or after any changes to the instrumentation, lab personnel, reagents, or if the technique is thought to be invalid. The process controls test the labeling procedure and the lysing step. If a computer-assisted analysis is used, the operator must independently check and validate all calculations and repeat tests on any values that are suspicious.

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1 Cluster of Differentiation (CD) is a controlled vocabulary (http://mpr.nci.nih.gov/prow/)

2 The FCS file format, universally adopted by instrument manufacturers, makes it possible to read data files from a variety of instruments. It consists of a text HEADER identifying the file type, a TEXT segment with various keywords to describe the specimen or condition under which experiment was done (no ontology referenced or used), and a DATA segment which contains the numerical data collected in a binary format. (http://murphylab.web.cmu.edu/publications/64-seamer1997.pdf)

Analysis

Gating (subsetting) strategies are a major contributory factor in result variability. An initial gate is set on a CD45 vs. side scatter dot plot to contain all CD45+ events (Figure 1). The events in gate R1 are then displayed on a CD34 versus side scatter and a second gate (R2) is drawn to include the cluster of CD34+ events. The third plot is obtained by plotting the events that fulfill the criteria of R1 and R2. Cells forming a cluster with characteristic low SSC and low to intermediate CD45 fluorescence are then gated on this third plot to produce region R3. Ungated data are displayed on a CD34 versus CD45 histogram to establish the lower limit of CD45 expression by the $CD34^+$ events. Finally, the events fulfilling all the criteria of all three gate (R1, R2, and R3) are then displayed on a forward light scatter versus SSC dot plot to confirm that the selected events fall into a generic "lymph-blast" region (R4). This region is precisely set to include events no smaller than lymphocytes by back scattering a small number of lymphoctytes from plot 1. Any events falling outside region R4 are excluded from the final calculation. $CD34^+$ cell determination must be performed in duplicate and the mean $CD34^+$ value used. The percentage of $CD34^+$ cells is determined by using the number of $CD34^+$ events expressed as a percentage of the $CD45^+$ events (both values derived from the mean of the replicate tubes – see above). The absolute CD34+ cell count in the sample is then calculated by multiplying this percentage value by the absolute total WBC as provided by the haematology analyser after correction to exclude nucleated red cells. Absolute counts should be calculated on all specimens tested and expressed as cells/ml. Harvesting of stem cells should commence when the peripheral blood CD34+ count is >10 cells/µl with the aim of having a final yield of $>2x10^6$ $CD34^+$ cells/kg. The results are reported as cells/ml and reported in a standard hard copy format. These forms and data files are kept for a minimum for 6 months and are stored electronically.

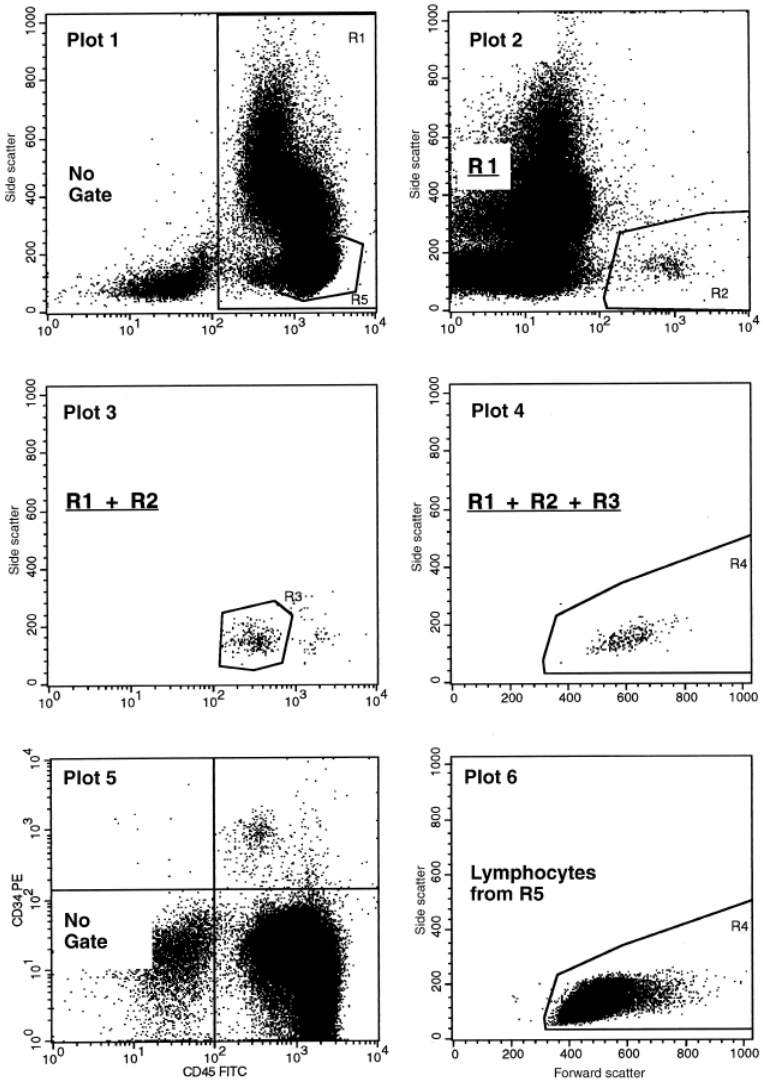

Figure 1. A peripheral blood sample analyzed for CD34+ stem cells.

Use Case #2 "Sorting"

Flow sorting extends gated analysis to isolate pure populations of cells. If it is possible to obtain cells of interest into a gate it is possible to isolate them into a test tube or multiwell plate. A flow cytometer for sorting is equipped with a mechanism (e.g., charged plates) to divert the sample stream and electronics that can determine within a few microseconds after a cell is detected if measured values fall within a gated region. As in Use Case #1 cells in solution are labelled with fluorescent signals. These signals can be derived from a specific biomarker such as a tumour antigen attached to an antibody that is labeled with a fluorescent signal or a recombinant DNA construct encoding modified proteins with a fluorescent signal. Multi-coloured fluorescence-activated cell sorters can selectively separate and collect homogeneous cells with identical phenotypic features in a collection tube in order to increase sensitivity. Collected cells are then lysed and RNA collected. This sample can then be used to determine the gene expression profile of a given cell type. A substantial amount of flow cytometry sorting is conducted for cells expressing green fluorescent protein (GFP).

For sorting, the sample stream is subjected to vibration by an electromagnetic transducer acoustically coupled to the flow chamber, and the pattern of droplet formation becomes stabilized in space and time. Cells labeled with the eight parameters are sampled at a rate of 10,000 – 40,000 cells/second.