
Materials and Methods

Quantitative Flow Cytometry

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Introduction

Quantitative flow cytometry, or quantimetry, is defined as the measurement by flow cytometry of the intensity of staining of cells, and provides an absolute value for the light intensity it measures. This is done by comparing cell fluorescence with an external standard.

Flow cytometry is employed mainly to enumerate cells, testing different parameters such as forward scatter (FSC) and side scatter (SSC) to assess the size and granularity of cells, and the fluorescence parameters to count the number of cells of interest that express a particular antigen stained by a monoclonal antibody on the membrane or the cytoplasm.

By using commercially available beads, it is now possible to quantify with a standard curve the amount of fluorescence relative to the peak channel obtained by flow cytometry. Given that the fluorescence settings of the instrument are kept unchanged when an unknown sample is run, it is possible to quantify its fluorescence intensity by using the value of its peak channel and by inserting the value into the standard curve. The number of fluorescence molecules per cell can then be estimated.

There are two units used to express the quantification of immunofluorescence: the ABC (antigen binding capacity) and the MESF (molecules of equivalent soluble fluorochrome).

Applications

The quantitation of fluorescence molecules by flow cytometry provides additional information useful for the precise characterization of cells. In haematology it has been employed for the identification of haemopoietic cell populations, both normal and leukaemic. In certain haematological malignancies, precursor cell-associated antigens can be under- or over-expressed on the malignant cells and can therefore be regarded as 'leukaemia-associated' when compared with normal counterparts. (Ginaldi *et al.*, 1995).

There is also a hierarchy of expression that is characteristic of different lineage, i.e. dim expression of CD4 in monocytes and brighter expression in T lymphocytes. In pathological conditions such as in infec-

tions, parasitic or viral, HIV, etc., the regulation of antigen expression is altered. Some antigens show unimodal expression on blood cell populations, and other antigens may be heterogeneously distributed with different densities. This may indicate functionally different subsets in relation to a particular differentiation stage of the cells.

The antigen density, when added to percentages or absolute counts of positive cells, exploits the informative value of the immunofluorescence test in the interests of defining both normal and abnormal differentiation pathways and subset compositions as well as signs of cellular activation. (Bibouke *et al.*, 1996).

In summary, quantitative flow cytometry has been used in the study of maturation processes, looking at differentiation antigens, in activation of neutrophils and complement receptors, in functional assays of adhesion molecules, in infections, looking at the expression of virus receptors, in oncogene products, drug receptors, steroids receptors, immunophenotyping and pathological situations.

Quantitative flow cytometry permits a more objective definition of positivity, provides a refined knowledge of haemopoietic differentiation and helps to ascertain malignancy. It also provides a useful clinical parameter for the detection of minimal residual disease (Farahat *et al.*, 1995).

Sample preparation

The samples should be prepared by the method of choice of the laboratory. There are two ways of staining the samples.

1. Direct immunofluorescence

In this procedure the monoclonal antibodies are used directly conjugated to the fluorochrome of choice: FITC, PE or third colour, and then washed and resuspended for reading in the flow cytometer.

2. Indirect immunofluorescence

In this method, the monoclonal antibody is not directly conjugated to a fluorochrome and a second incubation with a fluorescent antibody against the primary is needed to detect the reaction by flow cytometry. The secondary antibody is labelled with one fluorochrome: FITC, PE or third colour.

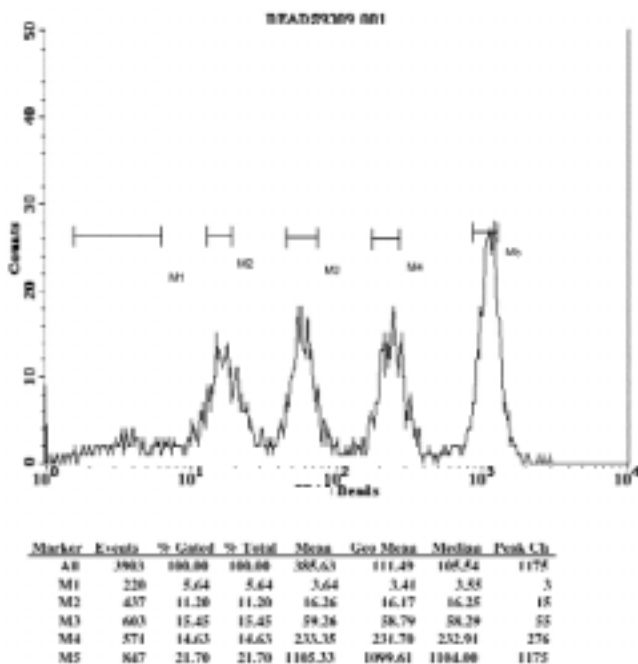


Fig. 1. FSC/SSC dot plot showing the gated bead acquisition.

Principle of the test

The choice of bead type depends on the procedure used for the sample preparation but the general principle for quantitating the fluorescence signal by beads is the same for both methods.

The beads are commercially available in a kit, which contains one tube with a mixture of four beads with four different predefined levels of uptake of fluorescence: one very dim, one very bright and two intermediates, and another tube with a blank, i.e. beads with no uptake of fluorescence; see Fig. 1.

There are three essential rules for performing successful quantitation:

1. The monoclonal antibody has to be applied at saturating amounts, both for the beads and for the cells in the specimen.
2. The same reagent, from the same company and at the same dilution, should be used throughout the experiment and for further analysis.
3. The instrument fluorescence setting should be maintained unchanged once the beads have been run, and the analysis of the unknown sample should be performed using the same setting.

Reagents

1. Direct immunofluorescence

QSC (quantum simply cellular): These are beads coated with goat anti-mouse (GAM) immunoglobulins; each level of the standard can bind to a certain amount of murine Ig. They are used to quantify direct immunofluorescence and they measure ABC (antibody binding capacity) when saturated with the same fluorochrome-conjugated monoclonal antibody as used on the cell sample. These beads require one calibration for each monoclonal antibody.

2. Indirect immunofluorescence

QIFIKIT: These are beads coated with predetermined amounts of CD5 monoclonal antibody in order to mimic monoclonal antibody-bearing cells. The secondary antibody used binds to the cells and the beads in a similar way; they require only one calibration per experiment (Poncelet *et al.*, 1996).

3. Direct and indirect immunofluorescence

FCSC (Quantum beads): These beads are coated with known molecules of fluorochrome; they are available conjugated to FITC or PE and measure the MESF. The fluorescence of the cells is compared with the standard molecules of fluorochrome-labelled beads.

4. PE conjugates

Quantibrite: These beads are used to calibrate the FL2 channel and to estimate the number of PE molecules bound per cell. This kit comes with the Quantquest software and PE-labelled McAb, which contain only 1:1 conjugates. The fluorescence intensity is given in units of antibodies bound per cell.

5. SCIFA: (stabilized cell immunofluorescence assay)

This system is based not on beads, but on stabilized cell batches. The advantage of this assay is that the McAb to be quantified is compared with stabilized cells with a known number of antigens on their surface and these are used as biological calibrators; this is a more physiological and accurate method than the use of beads. It is limited by the availability of stabilized cell batches and the results of validation in multicentre trials (Gratama *et al.*, 1998).

Protocol

1. Preparation of beads

Agitate the bead suspensions thoroughly in their bottles and dispense 50µl of this solution into a test tube. Add the McAb to the tube of beads at double the amount used for cell staining or, for a more accurate determination, titre the McAb with the beads to ensure saturation. Incubate for one hour at room temperature, wash twice with PBS before analysis and resuspend in Isoton.

2. Preparation of cells

The staining and preparation of cells is done according to the routine protocol of the laboratory; the difference in the quantification procedure arises during acquisition.

3. Flow cytometry

The stained beads are run first; at least 10,000 events should be acquired. Place an active gate on the microbead singlet population using the FSC and SSC dot plot; this will exclude the doublets during acquisition (see Fig. 2). Establish statistics regions in the fluorescence histograms for each of the five populations of microbead standards by applying a marker for each peak. Record the peak channel value for each of the regions and print statistics.

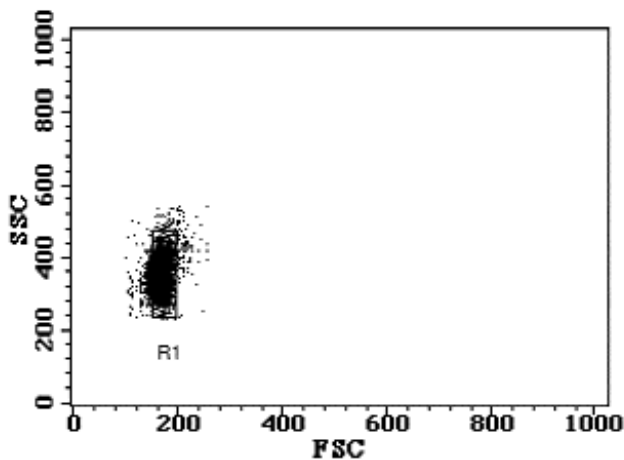


Fig. 2. Fluorescence intensity of blank and beads.

Maintaining the same settings for the FL1 and/or FL2 as those for the microbeads, run the tubes with the stained cells and determine their fluorescence peak channel values and print the statistics.

Calibration curve software

The standard calibration curve is obtained by plotting the values of the peak channels of the blank and the other four peaks obtained from the histogram against the known number of molecules of fluorochrome obtained from the supplier of the beads.

The peak value of the unknown sample is obtained by running the sample with the same fluorescence setting as the beads minus the peak value of the control tube (tube with the isotypic conjugate fluorochrome but with no primary antibody).

During the early days of quantification, the figures had to be transferred to database programs in order to produce a standard curve to obtain the value of the unknown sample and then to get the amount of fluorescence intensity either in ABC or MESF, the results of which were expressed in units of 10^3 molecules per cell. New tools were added to the quantification kits and relevant user-friendly computer programs are now provided with the beads. The data obtained from the cytometer are put into the program and a standard curve is produced automatically. These programs take into consideration the make of the instrument used, the voltage for that sample, the fluorochrome used, the supplier of the McAb, etc. The program calculates the ABC and/or MESF value of the unknown sample and stores the data; see Fig. 3.

Acquiring data on the flow cytometer

1. Acquiring the beads

The tube with the beads is acquired first in all cases where one experiment is sufficient for the quantification; this is the case for the Quantum beads and the QUIFIKIT beads. In the case of the QSC, where a tube is run for each McAb, the tube with the beads for that particular McAb should be run first, and then all

the other tubes with beads for the different McAb. The SSC voltage needs to be decreased more than that for cells in order to bring the beads into the FSC/SSC dot plot.

The instrument should be set up in such a way that the fluorescence signal of the tube with the blank beads is located in the region between 0 and 10^1 , and four other peaks of fluorescence should be seen along the axis of the relevant fluorochrome. Once the fluorescence voltage of the instrument is set up, these settings are maintained throughout the analysis of the unknown samples. In the case of the QSC, the settings for each McAb should be used accordingly.

2. Acquiring the sample

For the Quantum cellular and QUIFIKIT, one set of beads is used for the whole of the sample and different McAb, whereas with the QSC, one bead run fluorescence setting is used for every McAb. The samples for that particular McAb should therefore be run with the fluorescence settings for the run of beads corresponding to that McAb.

Pitfalls of the method

The volume of McAb needed to saturate the cell sample is not necessarily the same as that required to saturate the beads; a larger volume of McAb may be needed for the latter.

The position of the fluorescence setting of the blank may have to be negotiated and set up slightly lower or higher than the ideal situation just within the 10^1 limit. Some samples have very dim fluorescence and if the blank is set up too low, the intensity of the sample may be difficult to calibrate.

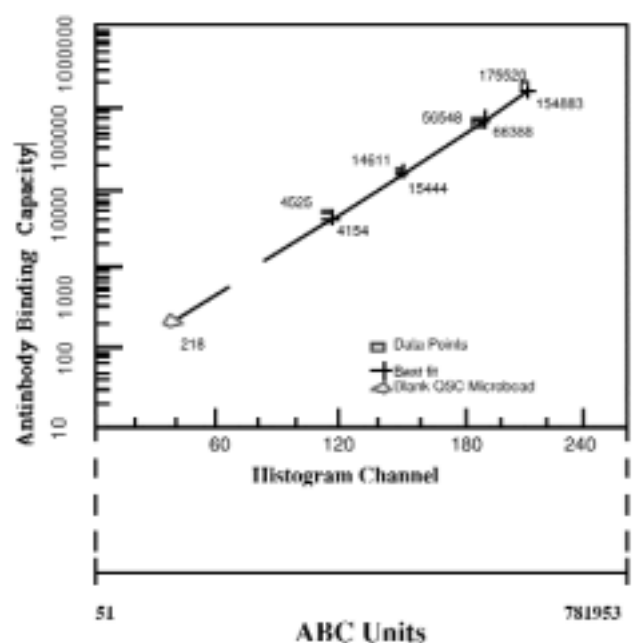


Fig. 3. Calibration curve using QSC beads.

