Flow Cytometry〈*G3-16-182*〉

(フローサイトメトリー〈*G3-16-182*〉)

Flow cytometry is a measurement technique for analyzing the optical properties of individual cells or particles dispersed in liquid and aligned by a fluidics system. In addition to obtaining morphological parameters such as the size and complexity of the internal structure of cells using scattered light, it is also possible to quantitatively obtain information about protein expression on cell surface and in cells and nucleic acid contents at a single cell level by staining cells with fluorescent-labeled antibodies or fluorescent dyes, etc. Also, by combining different fluorescent probes, information on multiple parameters can be obtained simultaneously. In the characterization and specifications of biotechnological/biological products, flow cytometry is used to evaluate the binding activity of a desired product to target cells, cell response, and the qualification of cultured cells used for bioassays.

1. Instrument and principle of measurement

An instrument used for flow cytometry (flow cytometer) generally consists of a fluidics system, a light source, an optical detection system, an electronic processing system (electrical pulse processing system), and a data processing system (Fig. 1).

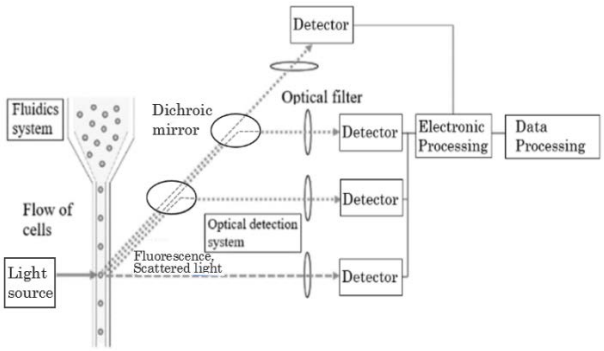

Fig. 1 Configuration of flow cytometer

In many flow cytometers, cell suspensions are transported by a fluidics system to a flow cell, where hydrodynamic focusing by a sheath fluid forms a thin stream of cells in a row, and the cells pass an observation point (laser interrogation point) one by one. An argon laser (488 nm), a helium-neon laser (633 nm), and diode lasers having various wavelengths are commonly mounted in combination, and appropriate light sources for the fluorescence to be detected are selected When cells pass through the laser interrogation point, light scattered in various directions is generated by the physical structure of the cells, and fluorescent dyes are excited to emit intrinsic fluorescence.

Scattering forward of the optical axis of a laser (usually within 20°) is called Forward Scatter (FSC), and the larger the cell, the stronger the FSC. Therefore, the relative size of cells can be estimated by measuring FSC. Scattering at 90° to the optical axis of a laser is called Side Scatter (SSC). The intensity of SSC is an indicator of the complexity of cell structure (the higher the complexity of the internal structure of the cell, the higher the SSC intensity), since the intensity of SSC is affected by the amount and type of intracellular granules, the morphology of nucleus and cell membranes, etc.

Depending on the type of a light source, fluorescent signals are produced by fluorescent substances contained in cells or by fluorescent probes (fluorescent dyes, fluorescent-labeled proteins, fluorescent proteins, etc.) used for specific analyses. Fluorescence emitted from cells is separated by an optical system and detected in individual channels. Optical filters include long-pass filters that allow fluorescence above a specific wavelength to pass through, short-pass filters that allow fluorescence below a specific wavelength to pass through, and band-pass filters that allow fluorescence in a specific narrow wavelength range to pass through. By combining these filters with a dichroic mirror placed at a certain angle to an incident light, fluorescence with a specific wavelength can be distributed to a target channel. The specificity of detection depends on the setting of an optical system, so the combination must be appropriate for the fluorescence to be detected.

The scattered light and fluorescence distributed by the optical filter are detected by a photomultiplier tube (PMT) or photodiode, and converted into voltage pulses. The voltage pulses detected by PMT can be amplified by applying voltage to the detector. There are two types of amplification methods, linear and logarithmic. In general, the linear amplification is used to measure the scattered light (FSC, SSC) of cells, while the logarithmic amplification is often used to measure fluorescence. A threshold is usually set for FSC to prevent the acquisition of data unrelated to experimental data, such as signals derived from particulate matters (contaminants such as cell fragments) contained in a sample. Signals that do not exceed the threshold are ignored by all detectors. The voltage pulse is an analog value, and in most flow cytometers currently in use it is converted to a digital value that can be processed on a computer by analog-to-digital conversion.

When two or more fluorescent dyes are used simultaneously to stain cells, a portion of the fluorescence spectrum of each dye may overlap, in which case each fluorescence detector detects the fluorescence emitted by the other dye in addition to the specific fluorescence from the intended fluorescent dye. To solve this problem of spillover, fluorescence compensation is performed. By using samples such as being stained independently with each fluorescent dye used in tests, the spillover of each fluorescent dye to other detectors can be calculated, and the data from which interfering signals are

selectively subtracted can be obtained. Amplified and compensated data for each parameter (FSC, SSC, fluorescence) obtained for individual cells through the above process are used for analysis.

2. Data Analysis

2.1 Data Display

Data obtained by flow cytometry can be displayed and analyzed in various ways (Fig. 2). One common display method is a histogram, which shows the signal intensity of one measurement parameter on the x-axis and the number of cells on the y-axis. Histograms are useful for evaluating the expression level and expression ratio of a specific marker molecule. In addition, a dot plot, which plots the signal intensities of different parameters on the x- and y-axes, is used to identify cell populations combined with two type of cell surface markers and evaluate the proportion.

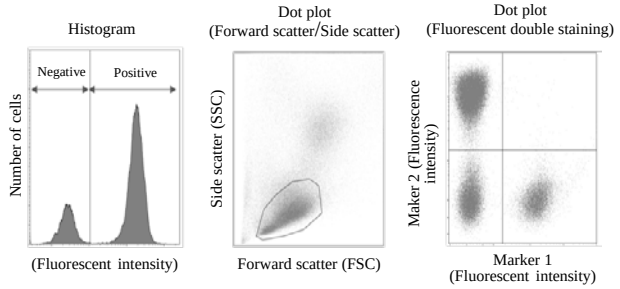

Fig. 2 Examples of data display

2.2 Gating

The acquired data may contain contaminants such as dead cells and cell fragments that are unnecessary for analysis, and signals derived from cell populations that are not the target of analysis, so gating is performed to analyze only target cell populations. Gating based on morphological characteristics of cells estimated by FSC and SSC is usually performed first. For example, dead cells or cell fragments with smaller FSC and larger SSC than live cells can be excluded from analysis by gating on an FSC/SSC plot. In addition, for the analysis of blood samples, lymphocytes and granulocytes can be distinguished and gated using FSC/SSC plots based on differences in cell size and complexity. In experiments using fluorescent-labeled antibodies against cell surface markers, cell populations expressing a specific marker molecule (e.g., CD3 in T cells, CD19 in B cells) can be gated and analyzed. Stepwise multiple gating can be set up using analysis software. For cell populations narrowed down for analysis by gating, the ratio of cells to which a fluorescent-labeled substance used in the test binds (e.g., the ratio of cells expressing a marker molecule recognized by a fluorescent-labeled antibody), the mean fluorescence intensity as an indicator of the amount of binding, and other parameters are calculated.

3. Points to note when measuring

3.1 Calibration of instrument

In order to obtain data with high reliability and reproducibility, instruments should be calibrated periodically. Many flow cytometers are provided with calibration software and reagents (usually fluorescent beads) by the instrument manufacturers, which are used to calibrate the instrument, and record the monitoring state of the instrument performance (variation in fluorescence intensity obtained from standard beads, setting of detection sensitivity, etc.).

3.2 Use of control samples

Control samples are used to identify background or nonspecific signals and to establish appropriate measurement conditions. Control samples are also used for routine test qualification (e.g., judgement of system suitability).

Unstained control: An unstained sample is used for gating cell populations to be analyzed, adjusting a detector based on a background due to cell autofluorescence, and setting a negative fraction.

Isotype control: When fluorescent-labeled antibodies are used, a control stained with an antibody that targets an antigen not present in cells being analyzed and is the same immunoglobulin subclass as the antibody used in the test is used to confirm that the staining observed is due to specific binding to the target antigen. Antibodies used for isotype controls should be labeled with the same fluorescent dye in the same ratio as the antibody used for testing. An isotype control is used to evaluate a background such as nonspecific bindings of antibodies and fluorescent dyes to cells and antibody bindings to Fc receptors on immune cells such as monocytes and macrophages.

Single-stained control: In the case of performing tests using multiple types of fluorescent dyes, a single-stained control is used for each fluorescent dye to evaluate spillover between the different fluorescent dyes and perform fluorescence compensation.

Fluorescence minus one (FMO) control: The FMO control is a control in which only one fluorescent dye is excluded from all fluorescent dyes used for staining. Using this control, it is confirmed by examining the spillover of other fluorescent dyes into the channel of the missing fluorescent dyes that fluorescence compensation is made correctly. The control can also be used to set up gating to determine negative/positive fractions.

Biological control (assay control): In addition to the controls for staining described above, prepare positive and negative control samples corresponding to a test to be performed. For example, in a test to measure changes in the expression of marker molecules associated with cell responses, untreated/unstimulated samples or samples with treatment known to certainly induce cell responses are used as controls. Measured data from these assay controls can be used to judge system suitability.

3.3 Setting measurement conditions

When measuring samples, select an optical system appropriate for fluorescence to be detected, and set detector sensitivity, gating, and fluorescence correction using control samples. Usually, the sensitivity of FSC and SSC are first adjusted so that cell populations to be analyzed is appropriately displayed in the FSC/SSC plot, and the cell populations to be analyzed is gated. Next, a histogram or dot blot is developed for fluorescence parameters to be detected, and the sensitivity of the detector is adjusted so that fluorescence detected in unstained controls and positive/negative controls is within a measurement range. The detected fluorescence intensity is the relative value that varies depending on the output of a laser, etc., and it is useful to set the sensitivity of the detector so that the fluorescence intensity of the control samples is within a predefined range to ensure reproducibility. When analyzing multiple-stained samples using multiple fluorescent dyes, use a single-stained control or FMO control to evaluate the spillover of each fluorescence to the other detectors, and set fluorescence compensation so that the spillover does not affect the analysis result. When calculating the ratio of positive fractions (expression ratio of marker molecules, etc.), gating is set so that positive and negative fractions can be distinguished using the fluorescence intensity of a control sample as an indicator. Set up the system suitability using an assay control, etc., and confirm that the measurement conditions are appropriate for routine testing.

3.4 Control of Cells and Reagents

Since cells and fluorescent-labeled antibodies used for staining are important reagents that affect the performance and results of tests, they should be managed in an appropriate manner by defining the criteria for evaluating their qualification. Since there is the possibility that characteristics of cells may change over the process of culture, a cell bank system should be established, and a culture method, the maximum number of passages and criteria for the condition of cells at the time of testing (cell viability, etc.) should be defined. When used in tests targeting a specific receptor, etc., the expression level of the target receptor should be defined and controlled as the specification. When performing the tests, it is also important to confirm that cells used in each test show expected cell responses using assay controls. Fluorescent labeled antibodies used for staining and cytokines used for cell stimulation should be used after confirming their suitability for the intended use. Since the specific activity of protein reagents may differ from lot to lot even if they are commercially available, when a lot is renewed, the old and new lots should be compared, and if necessary, the concentration of the reagent added should be adjusted for use in the test.

4. Examples using flow cytometry in biopharmaceutical testing

4.1 Evaluation of the binding activity of target substance to target cells

When a desired product exerts its pharmacological activity by its binding to a target protein on a cell surface (antibodies targeting cell membrane proteins, hormones/cytokines, etc.), the binding activity of the product to cells expressing the target molecule can be evaluated by flow cytometry. Cell-based binding assays have the advantage of evaluating the binding activity to target proteins on cell membranes under more physiological conditions, and are also useful for binding assays to multiple transmembrane proteins, which are difficult to purify recombinant proteins. On the other hand, nonspecific binding to non-target molecules present in cells used for the assay may occur, and the specificity of the observed binding should be considered.

As with binding assays based on other principles, either a non-competitive or competitive method can be used. In the non-competitive method, a fluorescent-labeled antibody against a desired product (e.g., fluorescent-labeled anti-human IgG antibodies against antibody drugs) is used to detect the binding of the product to target cells. In the competitive method, a sample is mixed with a fluorescent-labeled standard material or the equivalent, and added to target cells. The inhibitory activity of the sample on the binding of the fluorescent-labeled material to the target cells is measured. A dose-response curve is constructed from signals (mean fluorescence intensity) obtained by testing the dilution series of samples prepared at an appropriate dilution factor, and the dose that gives a signal equivalent to 50% of the maximum response (EC_{50} for the non-competitive method and IC_{50} for the competitive method) is calculated. To determine the relative activity to the standard material, a dose-response curve is prepared for the standard material and the sample, respectively, and the ratio of the EC_{50} or IC_{50} is calculated.

4.2 Evaluation of cell response

When an increase or decrease in the expression of cell surface marker molecules is observed as cell response to cell stimulation, the expression change can be quantitatively analyzed by flow cytometry. In addition to hormones and cytokines that induce cell responses through their receptors, flow cytometry is also used to evaluate the biological activity of humoral factors that induce cell responses and neutralizing antibodies that target their receptors. Cells that have been treated by adding a sample and culturing for a certain period of time are stained with a fluorescent-labeled antibody against a marker molecule to determine the ratio of cells expressing the marker molecule and the expression level of the marker molecule.

4.3 Qualification of cultured cells for bioassay

Flow cytometry is one of useful methods to confirm the expression of target proteins such as receptors in cells used for bioassay. Cultured cells may show heterogeneous gene expression patterns even in cloned cell lines, and their characteristics may change over the process of a culture period.

In addition, in cell lines generated by transfection to express target proteins, it is necessary to consider the possibility of loss or reduction of the target protein expression due to deletion or silencing of transgenes. The expression rate and level of a target protein should be measured by staining with a fluorescent-labeled antibody against the target protein to confirm that the expression rate and level of the target protein meet the predetermined criteria.