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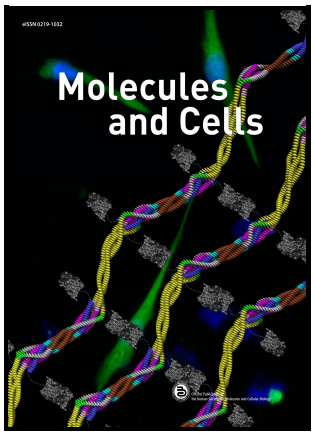

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Brief guide to flow cytometry

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ABSTRACT

Flow cytometry is a powerful analytical technique for measuring the physical and chemical properties of cells or particles as they flow through a beam of light in a fluid stream. It is widely used in several research fields for a wide variety of purposes. This article provides a concise guide to the principles and utilization of flow cytometry. Step-by-step instructions, from instrument setup to data analysis are intended to help researchers successfully apply flow cytometry in their studies.

Keywords:

Flow cytometry, Panel design, Compensation, Gating strategy, Fluorophore

INTRODUCTION

The discovery of green fluorescent protein, evolution of fluorophores, emergence of the concept of fluidics in the early 20th century, and advancements in optics have fostered the development of flow cytometry (Picot et al., 2012; Tsien, 1998). Diverse applications of flow cytometry in immunology, molecular biology, and clinical practice have further enhanced its power and effectiveness (Picot, 2012; Betters, 2015). Flow cytometry has a wide range of applications, including proliferation assays, fluorescence-activated cell sorting, immunophenotyping, cell cycle assays, apoptosis assays, identification of cell surface molecules, and nucleic acid analysis (Adan et al., 2017; Cossarizza et al., 2021). In this article, we provide concise guidance on how to utilize flow cytometry along with an explanation of the fundamental principles of the

technique. This guide aims to enhance the understanding and application of flow cytometry in various research areas.

MAIN BODY

Composition of flow cytometers

Flow cytometers comprise three major components: fluidics, optics, and electronics (McKinnon, 2018). The fluidics system is responsible for the appropriate delivery of the samples. The rapid flow of buffer, called the sheath fluid, aligns cells from the samples into a single-cell stream within a capillary of the flow cytometer. This single-cell stream allows the optics system to properly focus on each cell with excitation light, enabling precise analysis (Suthanthiraraj et al., 2013). The optics system comprises the following two elements: excitation lasers and detection components termed photomultiplier tubes (PMTs) (McKinnon, 2018). Laser irradiation excites fluorochromes conjugated to antibodies, and emissions from the fluorochromes are detected by the PMTs. Depending on the combination of lasers, a flow cytometer can analyze up to 50 parameters (McKinnon, 2018). After filtering out the background noise, the electronics system converts the detected signals into data that can be displayed for further analysis (Snow, 2004).

Preparation of samples

The first step in sample preparation is to understand the biological characteristics of the tissue and the cell type to be analyzed. For flow cytometry, it is crucial to identify

the markers of the samples. The Cluster of Differentiation (CD) nomenclature, sanctioned by Human Cell Differentiation Molecules, is used to name surface antigens on leukocytes and other cells (Zola et al., 2007). Additionally, identifying CD molecules on target cells that can precisely define the cell subset is essential before sample preparation.

After determining the cell-specific markers based on CD molecules, transcription factors, and other identifiers, the next step is designing the panel. Flow cytometry panel design involves the creation of combinations of markers with appropriate fluorophores and the establishment of a gating strategy. This step can be performed by the researcher or through commercial websites, such as BD Biosciences (https://www.bdbiosciences.com/en-us/resources/panel-design) or FluoroFinder (https://fluorofinder.com/flow-cytometry-panel-design/). For manual design, it is crucial to use spectrum-viewer websites (e.g., BD Biosciences; https://www.bdbiosciences.com/en-us/resources/bd-spectrum-viewer) to minimize fluorophore overlap.

Once the markers and fluorophores are determined, the staining procedure must be performed. Some subclasses of immune cells have Fc receptors, which couple with the Fc region of antibodies to induce effector functions (Roopenian et al., 2007). These Fc receptors are a primary cause of nonspecific binding of antibodies, especially on monocytes and macrophages (Andersen et al., 2016). In order to obtain better quality results, the use of an Fc blocker is recommended. After treatment with the Fc blocker, surface molecules can be effectively stained. When staining transcription factors or cytokines, cells must be fixed and permeabilized due to the intracellular localization of

these proteins. Fixation and permeabilization can be achieved with commercial kits, such as the Foxp3/Transcription Factor Staining Buffer Set (eBioscience™, Cat #00-5523-00) for transcription factor staining or the IC Fixation Buffer (eBioscience™, Cat #00-8222-49) for cytokine staining. In addition to the staining procedure, proper stimulation of the target cells is necessary prior to cytokine staining. This step may include various stimulants, such as phorbol 12-myristate 13-acetate and ionomycin, along with protein transport inhibitors like brefeldin A and monensin.

Additionally, the preparation of control samples for smear dyes or important markers with less distinct separation is highly recommended. One of the most convenient methods is the use of fluorescence minus one (FMO) controls, which include all fluorophore-conjugated antibodies, except for one, which serves as a negative control (Liao et al., 2016). Another form of control is isotype control. The purpose of the isotype control is to provide a benchmark for nonspecific binding of the antibody used in the experiment. It is therefore crucial to select an isotype control antibody that matches exactly in clone, chain (heavy and light chain subclasses), and fluorochrome (Hulspas et al., 2009). However, due to several limitations of isotype controls, FMO controls are becoming more widely used in current studies (Andersen et al., 2016).

When cells are excited by the excitation laser, they emit a spectrum of light. Given the nature of the spectrum, overlaps among different fluorophores are inevitable. This spillover can provide misleading data, resulting in false-positive or false-negative populations (Roederer, M, 2002). To address this issue, one can choose either automated compensation within the instrument, manual compensation using software such as FlowJo™, or both. For automated compensation, single-stained samples (e.g.,

one sample stained with fluorescein isothiocyanate [FITC] and another with phycoerythrin [PE]) and an unstained sample to account for autofluorescence should be prepared (Cossarizza et al., 2021).

Instrument setup and running

In this section, we describe how to set up and run the BD LSRFortessa manufactured by BD Biosciences and CytoFLEX LX flow cytometer manufactured by Beckman Coulter.

Instruction for BD LSRFortessa (Fig. 1A)

1. Run the BD FACSDiva software, create or log into an account, and create a new folder.

2. Create a new experiment.

3. Create a new specimen and a new tube.

4. Select the channels in use and adjust the voltages in the cytometer tab.

Note: While the initial fraction of the sample is running, adjust the voltages of each laser to ensure that most of the cells are within the plot area and import the appropriate compensation values based on the current voltage settings to improve the signal-to-noise ratio and correct for any spectral overlap or spillover between the channels (Rico et al., 2021).

5. Generate the gating strategies prior to starting.

Note: In most cases, the first plot to examine is the forward scatter (FSC) versus side scatter (Van den Bossche et al.) plot. FSC indicates cell size, whereas SSC reflects cell granularity (Scherer et al., 1999; Ramirez et al., 2013). Next, exclude doublets in the FSC width versus area plot to avoid misleading data (Donnenberg et al., 2015). It is also crucial to differentiate between live and dead cells, as dead cells often bind non-specifically to antibodies (Wing et al., 1990).

6. After loading the sample, determine the acquisition setup, acquire the sample, and record the data.

Instruction for CytoFLEX LX (Fig. 1B)

1. Run the CytExpert software, and turn on the instrument by clicking “Turn On” in the Cytometer tab. Initialize the instrument.

2. Set the channels in the settings menu, turn off the channels that are not in use, label the selected channels with marker names, and close the tab.

3. Set desired plots before loading the sample.

4. Determine the stopping strategy.

5. Click on the Run button after loading the sample.

6. While the initial fraction of the sample is running, adjust the voltages.

7. Record the data from the sample analysis.

8. Click on Next Tube and repeat the recording.

Analysis

Many software options are available for analyzing flow cytometry data. Among them, FlowJo™ is the most popular software. Gating strategies vary depending on the researcher or sample type. In this section, we provide a detailed description of one specific gating strategy using FlowJo™.

1. Gate the desired population in the FSC versus SSC plot based on the size of the target population (Fig. 2A).

2. Exclude doublets by gating out the larger population in the FSC area versus width plot (Fig. 2A).

3. Gate the live cells (Fig. 2A).

4. Narrow down the population from broadly categorized groups to the specific target population.

Note: Converting the logarithmic axis to a bi-exponential axis is often preferable, starting from gating for live cells. Logarithmic analysis has limitations for cells with low-intensity values. Some cells exhibit minimal fluorescence intensity and appear at the bottom of the axes, causing them to aggregate indistinguishably. In contrast, a bi-exponential axis, designed for flow cytometry data, can elevate the minimal fluorescence population instead of causing clustering at the bottom of the plot (Fig. 2B) (Herzenberg et al., 2006).

CONCLUDING REMARKS

Flow cytometry is a versatile and powerful tool with a wide range of applications in various fields of biology. By understanding its core components, namely the fluidics, optics, and electronics systems, researchers can optimize this technique to achieve accurate results. Although this paper focuses on flow cytometry, the principles of running samples and gating strategies can also be applied to cell sorting. The only difference between flow cytometry and cell sorting lies in whether the samples are discarded or collected after passing through the optics system.

Declaration of Competing Interest

The authors declare no conflicts of interests.

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AUTHOR CONTRIBUTIONS

Conceptualization: YS and YL; Writing-original draft: YS; Writing-review and editing: YL

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FIGURE LEGENDS

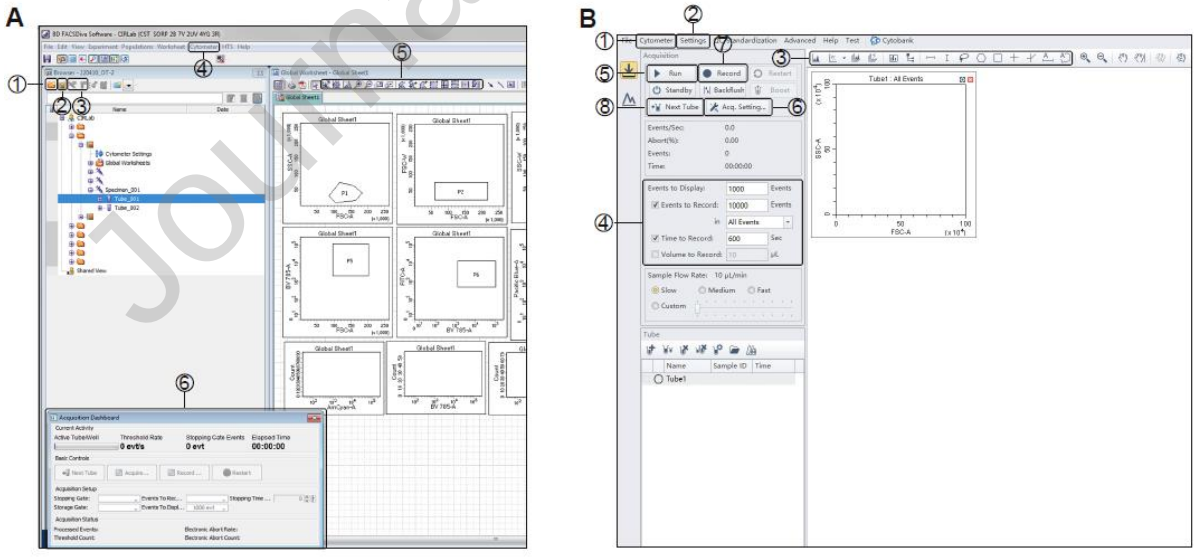

Figure 1. Instrument setup for BD LSRFortessa and CytoFLEX LX

(A) Steps for setting up the BD FACSDiva software. Each number in the figure corresponds to the steps written in the paragraph.

(B) Steps for setting up the CytExprt software. Each number in the figure corresponds to the steps written in the paragraph.

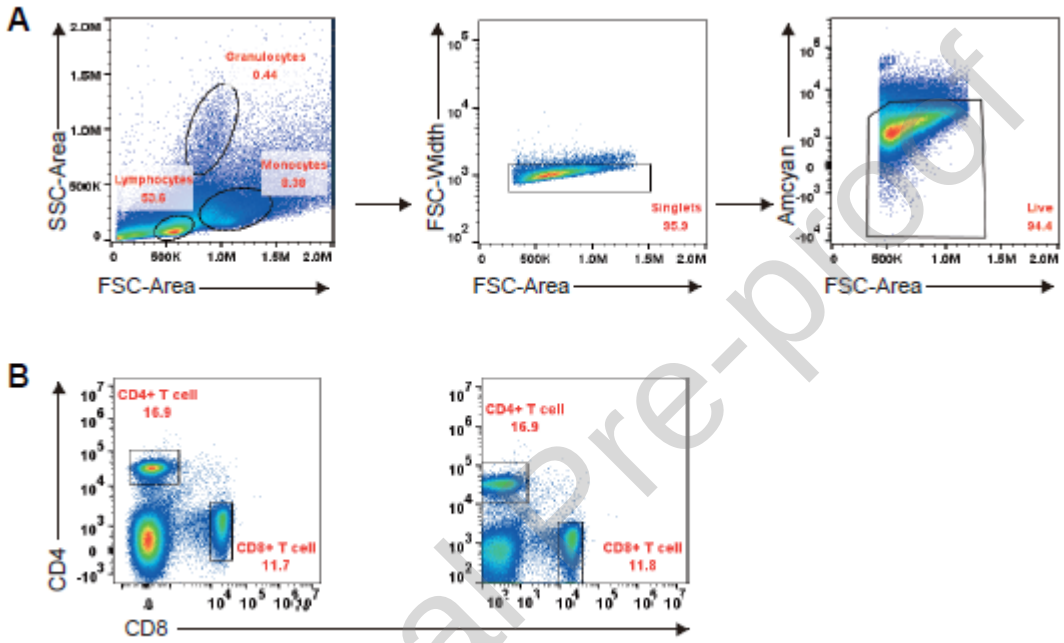

Figure 2. Analysis of flow cytometry data using FlowJo™

(A) Gating strategy for live lymphocyte populations from total mouse splenocytes. Excluding unnecessary populations helps remove cell debris or unwanted cells that might share markers with the target cells. Additionally, gating out doublets and triplets is crucial to avoid misleading populations. Excluding dead cells is also important to prevent false positives.

(B) Comparison of images between biexponential (left panel) and logarithmic (right panel) axes plots. Biexponential axes have an advantage over logarithmic ones

because logarithmic axes can cause populations with dim emission intensity to aggregate, making it harder to distinguish between them.