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Original article

Flow Cytometry in the Detection of Abnormal Cells and Cell Debris Based on the Expression of Cellular Markers

Eman Mohammed*, Najat Alasawad

[1]*Department of Histology, Faculty of Dentistry, Gharyan University, Gharyan, Libya*
[2]*Department of Physiology, Faculty of Dentistry, University of Tripoli, Tripoli, Libya*
Corresponding email. *eman.shiban@gu.edu.ly*

Abstract
Flow cytometry is a fundamental technique that is used in the fields of cancer biology and immunology to distinguish among the types of cells in mixed populations. As a diagnostic tool, it is also used by clinicians to identify abnormal cell populations associated with diverse diseases. The abnormal cell populations are identified through the specific binding of the antibodies to the marker of interest. Fluorescence measurements provide quantitative and qualitative data about cell surface receptors or intracellular molecules. The study examined the expression of CD49b, CD49c, and CD326 in the human colon cancer-derived cell line COLO 320 using immunofluorescence staining and flow cytometry. The cells were incubated with FITC-conjugated monoclonal antibodies and an isotype control, obtained from Serotec. Data were analyzed with the Cell Quest software. The FSC/SSC plot for the COLO 320 cancer cell line reveals two populations of cells with varying sizes and heterogeneity. Histograms show the expression of CD49b and CD49c, with moderate positivity for anti-CD49b and no specificity for anti-CD49c. CD326 staining in COLO 320 cells has two peaks due to subpopulations of varying degrees of commitment and origin. The CD326-positive population is gated, and unspecific binding is ruled out by the negative control.
Keywords. *Flow Cytometry, Cancer, Immunofluorescence.*

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Introduction

Flow cytometry is the technology that measures and analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The measurement and analysis are done simultaneously [1]. The measured properties include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity [2,3]. These characteristics are determined using the principles of light scattering and excitation, and emission of fluorochrome molecules. Light scattered in the forward direction, typically up to 20° offset from the laser beam axis, is collected by a lens known as the Forward Scatter Channel (FSC). The FSC intensity roughly equates to a particle's size and can also be used to distinguish between cellular debris and living cells. Light measured approximately at a 90 ° angle to the excitation line is called side scatter (SSC), which provides information about the granular content within a particle. Both FSC and SSC are unique for every particle, and a combination of the two may be used to differentiate among the types of cells in a heterogeneous sample [4]. Fluorescence measurements taken at different wavelengths provide quantitative and qualitative data about fluorochrome-labeled cell surface receptors or intracellular molecules. Once the signals are amplified, they are processed by an Analog to Digital Converter (ADC), which in turn allows plotting of events on a graphical scale [5].

Global efforts are still being made to prevent, diagnose, and treat cancer, which is the second greatest cause of disease burden and mortality worldwide, according to the World Health Organization [6],[7]. Flow cytometry is widely used in the fields of cancer biology and immunology to distinguish among the types of cells in mixed populations based on the expression of cellular markers [8]. Flow cytometry is a useful technique that is used in immunology, virology, molecular biology, cancer biology, and infectious disease monitoring, among other fields. As a diagnostic tool, it is also used by clinicians to identify abnormal cell populations associated with diverse diseases [9,10]. The abnormal cell populations are identified through the specific binding of the antibodies to the marker of interest. Further, the antibodies are artificially conjugated to fluorochromes to detect the markers [11]. When the cells are analyzed with flow cytometry, the cells expressing the marker, for which the antibody is specific, will manifest fluorescence [5]. EpCAM (CD326) is a multifunctional transmembrane glycoprotein with diagnostic, prognostic, and therapeutic relevance in cancer. Initially identified in colon carcinoma, it mediates epithelial cell adhesion and plays roles in signaling, migration, proliferation, and differentiation. Its expression is limited to epithelial tissues and related tumors, making it a useful biomarker.

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EpCAM detection is based on tissue morphology and staining, and it is commonly found in adenocarcinomas, metastases, malignant effusions, and cancer stem cells. Its overexpression is linked to advanced disease and poor prognosis [12].

Integrins, a family of transmembrane cell adhesion receptors, orchestrate a wide spectrum of cellular processes essential to the initiation, progression, and metastasis of solid tumors. By mediating interactions between cells and the extracellular matrix (ECM), integrins influence key biological events such as proliferation, migration, invasion, and survival. Their functional relevance across multiple cell types within the tumor microenvironment — including cancer cells, stromal fibroblasts, and immune infiltrates — underscores their pivotal role in tumor biology. Consequently, integrins have emerged as attractive therapeutic targets, with several strategies under investigation to disrupt their signaling pathways and attenuate tumor progression [13,14]. In the presented experiment, surface marker analysis was performed using direct immunofluorescence staining of colorectal cancer cells (COLO 320) using Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies. The flow cytometry technique was used to determine the level of expression for several cell surface markers, integrin α2 (CD49b), integrin α3 (CD49c), and epithelial cell adhesion/activating molecule (EpCAM) (CD326) were examined using FITC-conjugated monoclonal antibodies against these antigens.

Methods

Cell surface expression of CD49b, CD49c, and CD326 in the human colon cancer-derived cell line COLO 320 was determined by direct immunofluorescence staining and analyzed using flow cytometry. COLO 320 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cells were used between passage numbers 5 to 8 to ensure consistency and minimize phenotypic drift. At the time of harvest, cells reached 80–90% confluency.

For staining, cells were washed and resuspended in PBS/BSA buffer (phosphate-buffered saline, pH 7.4, containing 1% bovine serum albumin). A volume of 50 µl of the cell suspension was aliquoted into four cytometric tubes. Cells were incubated for 1 hour at 4 °C with 5 µl of FITC-conjugated monoclonal antibody or isotype control (IgG) as a negative control. Mouse monoclonal antibodies targeting human antigens CD49b, CD49c, and CD326, along with the isotype control, were obtained from Serotec (Oxford, UK) and handled according to the manufacturer's instructions. Flow cytometry was performed using a FACScan flow cytometer, and data were analyzed using CellQuest software (Becton-Dickinson, Heidelberg, Germany).

Results

Figure 1A shows an FSC/SSC plot for the COLO 320 cancer cell line, where each dot represents an individual cell passed through the flow cytometer. The concentrated population of cells of relatively equal size, based on side and forward scatters. As portrayed with the dot plot, there are two populations of cells. Further, the forward scatter data indicate that the size distribution of the cells varies, and the cells display heterogeneity with a large number of small cells/debris. (Figure 1 (B)) presents the histograms evaluating the total number of cells (y-axis) with the fluorescence (x-axis) relative to the expression of the marker of interest. In particular, the purple histogram presents background or non-specific fluorescence (negative control), which sets a discriminator at a point where any signal above is considered specifically stained with the antibody. The green and pink histograms present the expression of CD49b and CD49c, respectively. There is moderate positivity for anti-CD49b, while no specific anti-CD49c in the COLO 320 cancer cell line tested. In the COLO 320, there are many cells staining positive for CD326**.** The histogram for CD326-stained cells has two peaks due to subpopulations of varying degrees of commitment and origin. The CD326-positive population is gated (R1), as shown in Figure 1A. Unspecific binding was clearly ruled out by the negative control. Thus, the positive expression for CD326 and CD49b on COLO 320 cells was postulated.

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(A)

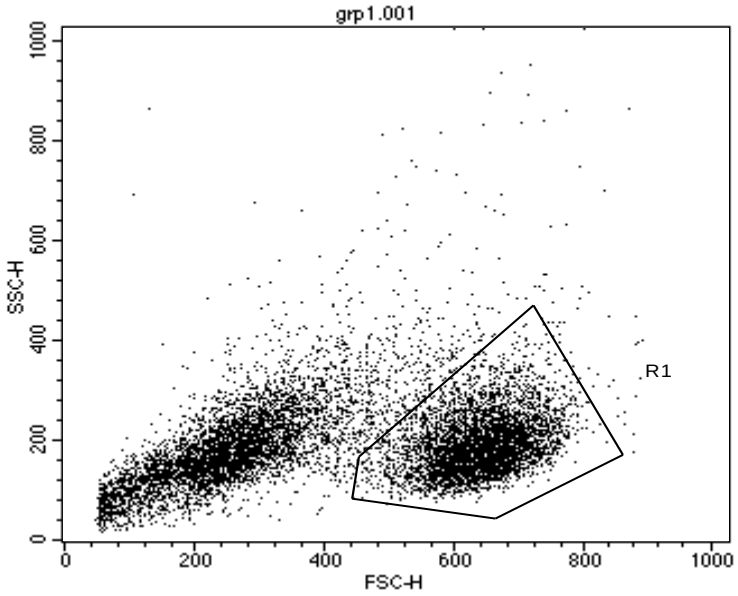
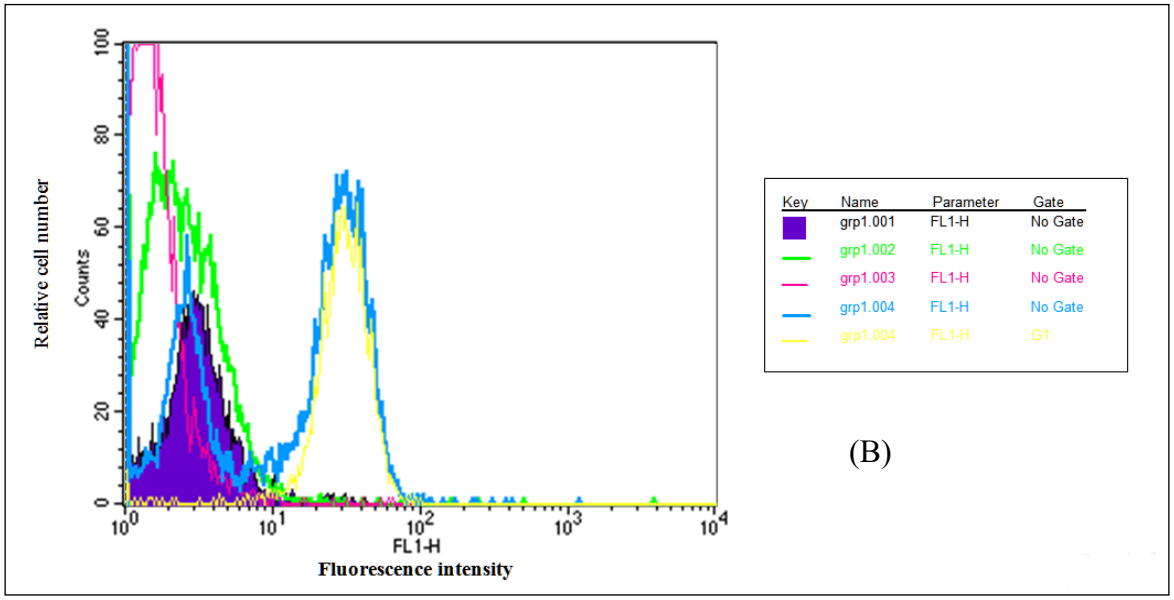


Figure 1. (A). Dot plot obtained from flow cytometric analysis of the COLO 320 cell line. (FSC-H) in the x axis represents the forward scatter, site scatter (SSC-H) in the y axis. The positive CD 326 population was gated (R1) using forward and side scatter.



(B)

Figure 1. (B). Surface marker expression characteristic in the COLO 320 cell line was analyzed by flow cytometry. Histograms showing surface markers and the differences in expression levels, relative cell count (y axis), and fluorescence intensity (x axis). Unstained control cells (filled histogram). The empty histograms represent cells stained with antibodies against the surface antigens, CD 49b (green), CD 49c (pink), and CD 326 (blue). The yellow histogram represents the positive CD326-gated population.

Discussion

Diverse cell surface markers are expressed on all normal cells. The markers are specific to types of cells and degrees of maturation. However, abnormal growth may interfere with the natural expression of markers, resulting either in over- or under-expression. Flow cytometry is widely used to distinguish between healthy and diseased cells [15]. The application of flow cytometry is referred to as immunophenotyping. Today, immunophenotyping is applied to aid the diagnosis of cancers and to monitor the effectiveness of clinical treatments. [16]. In the outlined experiment, the cell size distributions of COLO 320 cancer cells were assessed with flow cytometry as well. Based on the forward scatter

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data produced, the size distribution of the cells within the sample varied. In particular, the cells displayed a greater heterogeneity with a large number of small cells/debris and scattering of larger cells. Concentrated population of cells of relatively equal size based on side and forward scatter. Based on the surface marker analysis, cells are gated according to physical characteristics. For instance, subcellular debris and clumps are distinguished from single cells by size, which is estimated by forward scatter. In addition, dead cells have lower forward scatter and higher side scatter compared to living ones [11]. Although in this experiment, it was expected to discover one population of cells, it could be attributed to a different maturation stage for each population. Overall, gating proved to be an effective tool in establishing the population, which expresses the markers of interest [17]. In the expression analysis, the cells that aggregate by the antibody are shifted to the right on the forward scatter plot with respect to the control. The brightness would increase as the cells shifted to the right, and this is often associated with a reduction in the number of positive signals. In addition to this, any very small material on the scatter plot may represent debris from cells that have been damaged by handling. These can bind antibody non-specifically, and it is wise to draw an analytical gate to exclude them from the assessment [18]. From the histograms obtained, it can be seen that there are two peaks when anti-CD326 was used. The taller histogram (high cell number), which is shifted to the right, represents the gated population that overexpresses CD326, while the shorter histogram presents the other population of cells that bind anti-CD326 non-specifically. The small shift to the right that is seen with CD49b indicates an antibody which present in too low concentration to give a bright signal, or an antigen that is represented at low density on the cell surface, or an antibody binding with very low affinity [11].

Cell surface adhesion molecules (CAM), including integrins, comprise a group of transmembrane proteins that play an important role in cellular interaction, communication, signal transduction, cell motility, and migration [19],[20]. Integrins contribute to a number of pertinent intracellular functions, such as cell activation, proliferation, and apoptosis [21]. For example, in tumor tissue, integrins affect the metastatic process by collaborating in the promotion of adhesion to an extracellular matrix [22,23,24]. one study that used flow cytometric analysis of cell surface antigen expression on a human colon cancer cell line shows a high expression rate of CD49b (more than 70 %) and CD49c (40%) [25]. However, in this experiment, there were no expressions of both CD49b and CD49c antigens on the COLO320 cancer cells, and that may be due to epigenetic silencing, mutations, or cell-type-specific regulation. The difference can be attributed to another type of cancer cell line used in the presented experiment (COLO320) as compared to the (KM12SM) used by [26], or it can be attributed to a different stage of maturation.

The epithelial cell adhesion molecule (EpCAM, CD326) was originally identified as a marker for carcinoma due to high expression on rapidly proliferating tumors of epithelial origin. Normal epithelia express EpCAM at generally lower levels than carcinoma [27]. From the results obtained in this experiment, it is concluded that the flow cytometric data comply with previous results for EpCAM. Over-expression of EpCAM was observed in colon carcinoma; however, the clinical significance of the aberrant expression suggests its involvement in colorectal metastasis [28].

Conclusion

In conclusion, flow cytometry, as a technique that uses antibodies that bind specifically to the marker, provides a tool to distinguish different cell types in mixed populations based on the expression of cellular markers. The overexpression of these markers suggests their possible involvement in colorectal cancer metastasis.

Conflict of interest. Nil

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