

THE TECHNIQUE OF FLOW CYTOMETRY IN DIAGNOSTIC RESEARCH

Arkadiusz Macheta¹, Katarzyna Radko¹, Agnieszka Szymczyk², Monika Podhorecka¹

¹ Department of Hematooncology and Bone Marrow Transplantation, Medical University of Lublin, Al. Raławickie 1, 20-059 Lublin, Poland, e-mail: arek.macheta@gmail.com; katarzyna.radko@gmail.com; monika.podhorecka@onet.pl

² Students Scientific Association at Department of Hematooncology and Bone Marrow Transplantation Medical University of Lublin, Al. Raławickie 1, 20-059 Lublin, Poland, e-mail: agusia_szymczyk@wp.pl

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ABSTRACT

Flow cytometry is a technology that simultaneously counts and then examines multiple physical and/or chemical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell scatters incident laser light and emits fluorescence. One of the most significant applications is immunophenotyping of cells - the most important tool in diagnosis and monitoring haematological disorders, such as acute and chronic leukemia, lymphoma, monoclonal gammopathy, myelodysplastic and myeloproliferative diseases.

Keywords: flow cytometry, immunophenotype, hematooncology.

INTRODUCTION

Flow cytometry technique dates back to the 1930s, when *Science* magazine published an article on the possibility of using a photoelectric sensor in counting the cells flowing through a capillary tube. In fact, though, ten or more years had passed from when the idea was conceived to when the design works began [9, 16]. Originally, cytometric tests were focussed on detecting traces of biological warfare toxins, which explains the commitment of the American Army to develop this technique during the Second World War. The prototype of a modern-day cytometer was composed of a basic dark-field flow chamber, with the brightest lamp available at that time – the Ford headlight – providing the source of visible light [15]. Further design and methodological research focussed on numerous areas of perfection and the use of flow cytometry. The most interesting is the fact that following the recent terrorist attacks history is making a full circle – now the American Army is again interested in the usefulness of the

technology discussed for the detection of microorganisms in the air [2].

THE FLOW-CYTOMETRY TECHNIQUE

Flow cytometry is an advanced technology involving the quantitative and qualitative multi-parameter assessment of individual cells [3]. The analysis consists of the identification of surface or intracellular antigens by means of specific monoclonal antibodies conjugated to fluorescent compounds, the so called-fluorochromes. Cells marked with these compounds are irradiated with a laser beam while they flow through the measurement chamber, as a result of which they disperse the light and emit it through activated fluorochromes. By means of a system of photomultipliers, light impulses are converted into electrical impulses, which in turn are subjected to analysis using a computer system compatible with the cytometer [3, 7, 17]. Coordination with the main construction systems of the flow cytometry

eter, that is with the hydraulic, optical and electronic systems, ensure the great measurement potential of this complex measuring apparatus. This technique is a rapid and sensitive method for the diagnosis and monitoring of the clinical course of diseases of the haematopoietic system [3].

The Hydraulic System

The role of the fluid-transfer system is to transport the suspension of the examined cells to the measurement chamber. When the suspension flows through the chamber it is surrounded by an isotonic sheath fluid and prevents the cells from adhesion. Measurements are conducted during the transfer of the compounds suspended in a stream of fluid through a tapering microcanal. In line with the principle of hydrodynamic focusing, the construction of the cytometer enforces the laminar flow of the analysed cells and their concentration, which is low enough to allow them to flow one by one into the area irradiated by a focussed light beam, exactly in the middle of the hydraulic canal (Fig. 1) [3, 11].

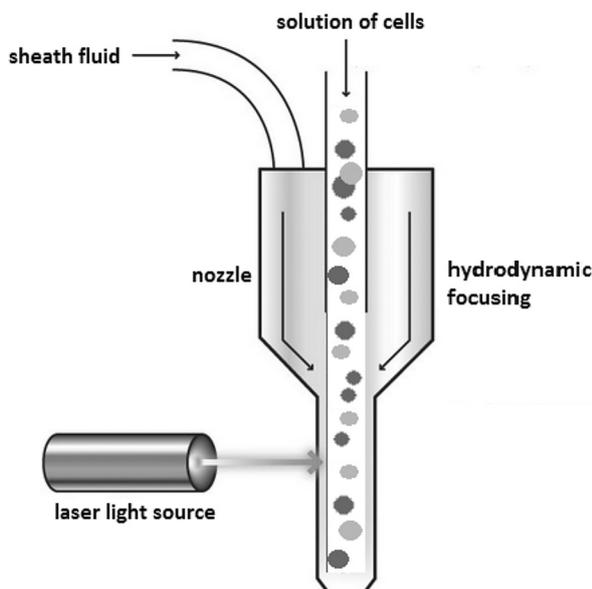


Fig. 1. The hydraulic module in flow cytometry

The Optical System

The role of the optical system is to generate and collect light signals – i.e. dispersion and fluorescence. Initially, the beam of light is pointed at the cells flowing through the chamber, perpendicularly to their flow direction. The type of the source of light used depends on the manufacturer - these are in the majority fixed-wavelength la-

sers, e.g. argon ion (488 nm), krypton, or helium-neon lasers (Fig. 2.) [7, 11, 13]. The mercury-vapour lamps that were commonly used in older types of cytometers (ICP, Phywe) for the production of light beams are nowadays supported by laser technology. Increasingly more devices have more than one source of light [8, 13].

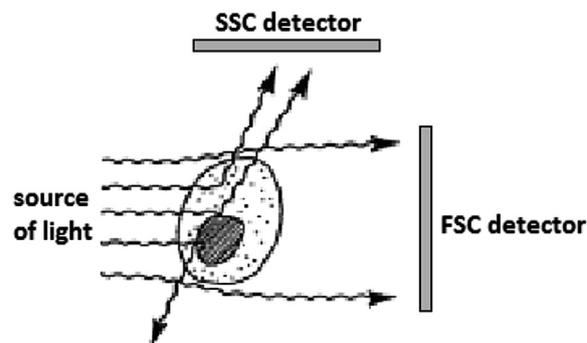


Fig. 2. The optical module in flow cytometry

The Electronic System

The electronic system in flow cytometry converts light signals into electronic impulses, which in turn will be converted into graphic signs (PMT). It comprises devices for converting signals into values, i.e. into digital images, and a computer system for storing and processing the resultant data. It contains a set of photomultipliers, signal amplifiers and an analogue-digital converter. The reception of signals is by means of forward scatter (FSC) detectors, side scatter (SSC) detectors and fluorescence detectors, because the cells diffract and disperse the light when they cross the laser light beam. The intensity of light dispersed at the edges of the analysed cell in the same direction as the laser beam enables the FSC detector to divide the cells according to their size. Based on the diffraction of light at an angle of 90 degrees towards the laser beam on the cell membrane and in the intracellular structures, the SSC detector divides the cells according to their shape and granularity [10]. After marking the cells with fluorescent dyes the intensity of the fluorescence can also be measured. The laser light beam forces a brief transfer of electrons to an orbit of the atom with lower energy, i.e. with a wavelength greater than the length of the light wave. The phenomenon of fluorescence is the result of electrons transferring back to their original orbit. It is important to employ fluorochromes with absorption maxima within the range of the wavelength of the laser in

our cytometer in the tests [10, 13]. Subsequently, light intensity is measured using appropriate detectors and the resultant signals are amplified by the photomultipliers, formed and send to the computer.

The Computer Presentation of the Results

The data analysis module allows comprehensive data processing, with several types of charts available for the visualisation of results:

- one-dimensional charts (histogram)
- two-dimensional charts (dot plot (Fig. 3), density plot, contour plot)
- three-dimensional charts (perspective charts)

During the analysis, it is possible to gate a group of cells, to subject the group of cells to quantitative assessment in every area and the calculation of various statistical values, which means that they are repeatable and accurate, collected in a short period of time and based on the analysis of several thousand cells [6].

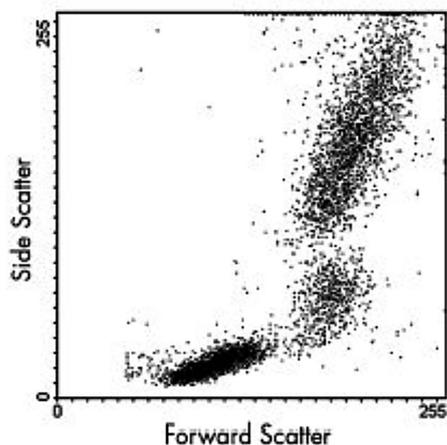


Fig. 3. Two-dimensional dot plot for peripheral blood

THE APPLICATION OF FLOW CYTOMETRY IN HAEMATOLOGY

Flow cytometry enables the assessment of the morphological features of cells with the determination of their maturity level and diversity. In clinical diagnostics, this method is mainly applied in the test concerning diseases of the haematopoietic system – thanks to developed panels of antibodies, flow cytometry helps not only to assess the immunophenotype of proliferating cells but also to establish their origin. Medicine employs a panel of monoclonal antibodies enabling the assessment of the affiliation of cells to a line

and stage of haematopoietic development. The classification of affiliation to the line and the recognition of the differentiation stage are based on establishing the co-expression of some antigens and the phenomenon of the disappearing group of antigens with others emerging in the subsequent stages of normal cell maturation. The earlier stage of differentiation and maturity is disrupted, the more difficult it becomes to diagnose the cell origin is only on the basis of morphology [4, 5, 6, 19] (Table 1).

Table 1. Basic immunophenotype markers of peripheral blood cells [12]

CD45	All leucocytes
CD19, CD20	B lymphocytes
CD3, CD7	T lymphocytes
CD4	Helper T lymphocytes
CD8	Suppressor T lymphocytes
CD56	NK cells
CD25, CD71, CD69	Lymphocyte activation
CD13, CD33	Myeloid differentiation
CD15	Granulocytes
CD14	Monocytes
CD34	Stem cells (progenitor cells)

The technique of flow cytometry has many advantages, including the possibility to perform examination using a small amount of biological material, even without their initial treatment, and the concurrent identification of surface and intracellular markers [3, 14]. It enables testing in the *in vitro* setting, whereas it is possible to run analysis only of the cells in the suspension. Therefore, the most convenient materials are blood or body-fluid cells. However, the analysis of solid-tissue cells is also viable after precise fragmentation and obtaining single and undamaged cells. It is also crucial to prepare a sample properly, especially in the case of assessing cellular functions. The most reliable results of analyses are obtained after testing freshly-taken material and, if it is necessary to store samples, cells should be fixed and stored in conditions preventing them from degradation; otherwise non-specific bindings of damaged dead cells with monoclonal antibodies may occur and, consequently, false results may be generated. Thanks to its broad applicability, flow cytometry is considered to be the basis for diagnostics and disease monitoring. Moreover, it is useful in assessing phenotypes in physiology, as it facilitates quantitative and qualitative assessment of antigen

expression in various cell populations, even in very heterogeneous ones. The basis for routine cytometric diagnostics includes the identification of the presence of particular subpopulations and their physical features, assessing the absolute amount of population cells tested and the percentage of particular populations. There are conditions where assessing immunophenotype is helpful and sometimes even essential to recognise or monitor a disease, as exemplified by:

- proliferative disorders (especially lymphomas and leukemias),
- immunodeficiency syndromes (congenital and acquired),
- organ transplantations,
- autoimmune diseases,
- diseases treated with immunosuppression,
- cancers [2, 19].

The employment of this technique is not only limited to determining immunophenotype, as exemplified by its application in medicine involving the testing of platelets of the whole blood, which helps observe accurately the status of thrombocytes in haemorrhagic diathesis, or hypercoagulability or assessing the effectiveness of antiaggregation treatment, or assessing the functions of phagocytes through the assessment of phagocytosis, chemotaxis, or producing reactive oxygen species, or identifying chromosomal mutations along with the *in situ* hybridisation technique (FISH) [1, 2].

This technique is unquestionably of a great importance in the diagnosis of lymphoma and leukemia. Cell subpopulations are characterised in detail and classified based on cell differentiation, surface and cytoplasmic antigens. This brings unique possibilities for describing cancer and its biology on the basis of molecule expression in the process of neoplasia and provides opportunities to identify the stage of the development of the cell on which the cancer is growing, and the degree of its maturity, and helps determine the functional features, such as cytotoxicity or the production of immunoglobulins. As regards of lymphomas and leukemias, flow cytometry is for:

- providing detailed diagnosis,
- confirming/excluding residual disease,
- as the method of choice for monitoring anti-cancer therapies,
- controlling cell transplants,
- assessing the regeneration of the bone-marrow microenvironment [18].

CONCLUSIONS

The flow cytometry technique developing is the result of cooperation between many research teams, especially biologists and engineers. The first devices enabled cellular analysis with regard to size and granulation. At present, the modern cytometer is even equipped with several dozen detectors for collecting data, and several lasers with various light lengths and complex optoelectronic systems. Contemporary equipment provides possibilities for several simultaneous, multicoloured testing of up to 10 fluorescent markers and two parameters of dispersion in a stream of cells transported at the speed of up to 70000/sec. So, we are involved in significant analytical options burdened with the risk of error and difficulties in comparing the obtained data. Particularly important are the parameters of the flow cytometer itself. Its model and type of software used should be included in the methodology. The efficiency of laser lines and filters of emissions used with the appropriate fluorochromes should be also employed. The use of many dyes increases the risk of disruptions resulting from overlapping spectra. This can be avoided thanks to the so-called compensation, i.e. narrowing the spectrum of detection to a very narrow wave range. This function is set into a cytometer during the collection of data and it should be included in the description of an experiment, e.g. the size of samples, statistical data and the minimum detection threshold. The lack of possibility to assess the topographical origin of cells is an impediment to the diagnostics and is a form of disadvantage for flow cytometry. Sometimes the necessity to deliver live and undamaged cells is also problematic. The technique of flow cytometry is becoming more and more useful in diagnostic research in haematology and other fields of medicine, although it is also commonly applied in many various areas of biological research.

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