FROM CAPILLARY GLASS TUBE TO GOLDEN ERA OF FLOW CYTOMETRY. DEVELOPMENT AND APPLICATION OF FLOW CYTOMETER

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S u m m a r y. Recent years have witnessed rapid development in the field of medicine, in terms of prevention, diagnosis and treatment of severe diseases. The ongoing progress has set high standards in the field of diagnostic techniques. Cytometry, which dates back to 1934, is currently experiencing its boom. It is difficult to imagine that the prototype of the cytometer was an ordinary glass tube filled with a liquid with cells passing through. Nowadays, flow cytometers are highly specialized devices that allow to perform necessary tests in the field of diagnostics of congenital and acquired immune deficiencies, cancer, leukemia, lymphoma and many others. We can therefore describe cytometry as a unique diagnostic method, with a significant progress made over the 80s.
K e y w o r d s: flow cytometry, modern cytometer,

INTRODUCTION

Flow cytometry is a future-oriented research and diagnostic technique used in many fields of medical science and biotechnology, which allows to describe cell population, that may be even less than 1% of the tested sample [10]. This analytical method enables fast quantitative determination of populations and subpopulations of cells, their morphological features, action state, and with the use of monoclonal antibodies combined with fluorochromes - assessment of immunophenotype at the presence of differentiated antigens on cell membrane surface. Flow cytometry also enables analyzing certain molecules, structures, cytokines and other intracellular substances. With its use and simultaneous measurement of light scatter and fluorescence signals emitted by the excited fluorochromes, multi-parameter analysis of biophysical and biochemical properties of single cells and their components [3, 4] becomes possible.

Cytometric analysis is performed using flow cytometer. There are two basic types of flow cytometers: analyzers, and more advanced - sorters. The former enables the assessment of the expression of surface and intracellular antigens, enzyme activity, as well as gene expression and transcription of mRNA for various cellular products. On the other hand, sorters allow not only the analysis of the flowing cells, but also their sorting. This method consists of tilting the flowing particles according to their differences in electrical potential and fluorescence. The purity of isolated cells in the most advanced sorters is > 99 %. As a result, sorters are used for the isolation of rare cells, such as captured in blood tumor cells, fetal erythrocytes and genetically modified cells. Moreover, they are used in transplantation and in vitro fertilization [2]. It is worth noting though,

that modern flow cytometers measure up to several dozens of parameters which, combined with the ability to sort specific cell populations for further diagnosis, gives great opportunities unavailable in other methods [5]. Sorting can be done into tubes, microtiter plates, or directly onto the glass slide.

FLOW CYTOMETER METAMORPHOSES

The first reference of cytometer dates back to 1934, when Andrew Moldavan published an article: Photo-electric technique for the counting of microscopical cells in Science magazine. In his project the cytometer was in the form of a capillary glass tube [16], through which he passed up the cells, and the light signal was analyzed by a photoelectric sensor [2]. Unfortunately, the idea never „saw the light of day" and the device was not constructed. Further references in which Gucker presented the first reports of cytofluorimetric detection of bacteria in aerosols [5] appeared in 1947. These studies started during World War II, in order to identify the bacteria in the air in the event of biological weapon use [18]. An important role played the flow chamber, where the air molecules were analyzed in a dark field, when illuminated with visible light of the car reflector from a well-known company - Ford. That was just the beginning of the development of flow cytometry. By 1970 there were only two companies dealing with cytometers: American Bio/Physics Systems founded by Lou Kamentsky and German - PHYWE AG. The following years experienced a boom of new and improved cameras. Soon enough, Hemalog D - the first hematology cell counter, cell sorter FACS by Becton Dicknison, TPS1 and Coulter EPICS series appeared on the market. The merge of Bio/Physics and Johnson & Johnson took place in 1976 and Ortho Diagnostics Systems was formed, which together with Coulter and Becton Dickinson were leading companies in the production of cytometers with cell sorting. Cytomation was the new cytometric company that appeared on the market in 1988 and began selling MoFlo sorters in 1994. At the moment, the market leaders were BD Biosciences, Beckman Coulter and DakoCytomation. In addition to the three, many new and prosperous companies have offered their cytometers [2, 15, 17].

CONSTRUCTION OF FLOW CYTOMETER

The flow cytometer consists of three basic systems: hydraulic, optical, electronic, and a light source and a data analyzing system. The task of the first is to produce a vacuum in the container with focusing liquid and in the cell suspension tube. Due to that, the analyzed molecules form a thin stream and pass through the measurement point at an appropriate speed. The light source, which is the laser hitting the particles, produces a dispersion and fluorescence signal. The optical system consists of shaping and focusing the laser beam on the cell system, as well as focusing optics. The function of the latter is to filter out individual wavelengths and focus the light rays on photocathode of the detectors. The electronic system is responsible for processing light signals into electrical impulses, and owing to data analyzing system, it is possible to analyze the results statistically. [2, 4, 8]

FLOW CYTOMETER: THE PRINCIPLE OF OPERATION

The principle of operation of a cytometer is simple, yet the individual requirements of the components are high. For a reliable, repeatable and accurate result, a stable flow, precisely collimated optics and high-end electronics must be provided by the analyzer.

The cells labeled with antibodies are aspirated from the sample, arranged in a thin stream and pumped under pressure to the measuring chamber where they pass through individually, and are irradiated with a precisely focused laser beam. The laser light is refracted, scattered at different angles, reflected, and at the same time excites the fluorochromes attached to the cell. With the use of appropriate detectors, the light intensity is measured and the created electrical signals are amplified, shaped and transferred to the computer for further processing, storage and analysis. The cells pass through the measurement zone at high speed - from a few hundred to several thousands per second. As already mentioned, the measurement of the flow cytometer is based primarily on the detection of the scattered light on the cell and emitted by the excited fluorochrome. Two detectors are used to measure the scattered light. The first one, FSC-Forward Scatter front detector detects scattered light according to the laser beam direction, which grows with the particle size. Therefore, FSC detector provides information about the cell size. Another one, i.e. SSC Side Scatter detector registers the dispersion at 90° which depends on the shape of the molecule, as well as its refraction and reflection indices. Thus, SSC separates the cells in terms of shape and internal granulation [3, 4, 12, 19].

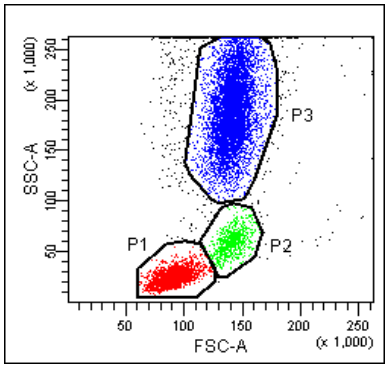
For cell immunophenotyping, monoclonal antibodies labeled with fluorochromes, i.e. colorants, are used, which show fluorescence when excited by laser

light. Each fluorochrome has a specific fluorescence of a particular spectrum and maximum wavelength. The light of different wavelengths is separated by means of special filters or dichroic mirrors, and then directed onto appropriate detectors. Individual detectors are responsible for the capture of fluorescence within a specific wavelength range [4]. The intensity of the emitted fluorescence caused by excitation of laser light with a wavelength close to the maximum absorption for a given fluorochrome is directly proportional to the amount of the cell component labeled with this fluorochrome [5, 9]. Additionally, having analyzed the shape of fluorescence impulse (signal width, its surface) it is possible to distinguish a single cell from aggregated cells, i.e. doublet discrimination [3, 7].

The results can be graphically presented in the form of charts:

- dimensional (histograms) (Fig. 3),
- two-dimensional (dot, density, contour) (Fig. 1, 2),
- three-dimensional (perspective).

The analysis is possible due to the software which, together with the computer system, is an essential and integral part of each camera, enabling simultaneous gating, quantitative assessment of cells in each region, calculation of statistical value, etc. The populations, which show specific parameters, can therefore be distinguished and characterized. [7, 13]



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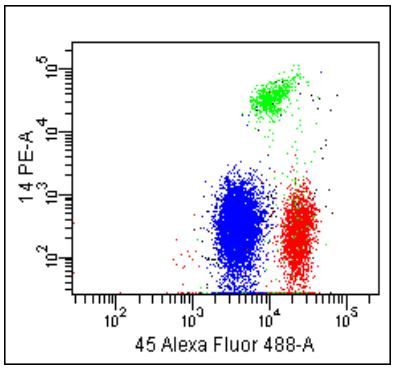

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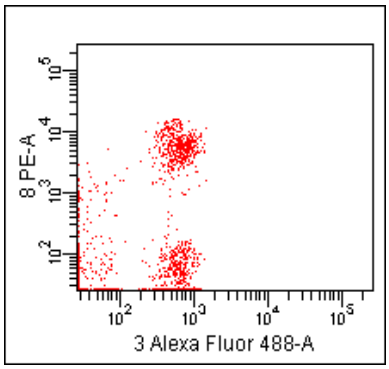

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Fig. 1. Examples of dot plots. Each point on the chart corresponds to one cell with appropriate values. Fig. 2a shows the distribution of cells, depending on FSC/SSC morphology, gate P1 corresponds to lymphocytes, P2 - monocytes, and P3 - granulocytes. Fig. 2b shows the distribution of cells, with regard to intensity of CD14 expression vs. CD45, while 2c shows the expression of CD3 and CD8 at the lymphocyte gate (P1).

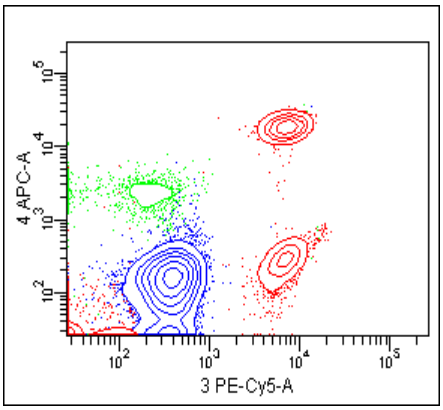

Fig. 2. Contour chart. Number of cells from appropriate gates expressed by contour lines (red color corresponds to lymphocytes, green - monocytes, blue - granulocytes).

Fig. 3 Histogram. The X-axis - the expression of CD3 antigen on a logarithmic scale (red color corresponds to the lymphocyte gate, green - monocyte, blue - granulocyte). The Y-axis shows the number of cells in each channel.

PHYSICAL BASIS OF CYTOMETRY

Flow cytometry is based on physical phenomena such as hydrodynamic focusing, excitation and fluorescence.

Hydrodynamic focusing - slow flowing cell suspension (v=2mm/sec) is taken by a fast flow-

ing (v=8m/sec) sheath fluid within a special nozzle. The cells flow laminarly as a thin stream comprising a row of individual cells. The sheath fluid flows faster than the fluid with cells.

The electron **excitation** in the molecule is associated with a change in the electron state. The electrons in the molecule normally reach an oscillating zero-level of the ground state S_0 (not excited), i.e. singlet state (S=1/2-1/2=0). Having absorbed a quantum of light of appropriate energy, the electron may be transferred to any oscillating level v_0, v_1 corresponding to the state S_1, S_2, etc. In S_1 excited state the electron's residence time is short - within the range of 10^{-6} sec., while at higher levels it is even shorter - within the range of 10^{-11} - 10^{-14} sec. After this time, due to nonradiative energy dissipation, all electrons from the higher singlet state change to oscillating zero-level of S_1 state. This phenomenon is called internal conversion. The electron that is in S_1v_0 state may return to one of the oscillatory levels of state S_0 via the radiationless decay or photon emission. A consequence of the Franck-Condon principle is a more frequent occurrence of such decays, which have their origin in the range of maximum probability of density of the atomic nuclei position. When the distance between the nuclei in the excited state increases, the most probable are various decays and the absorption curves (including fluorescence) of a different shape.

Fluorescence is the phenomenon characterized by the emission of light by a molecule as a result of its excitation (absorption of radiation), and the return of the electron from v_0 oscillating state S_1 to any oscillatory and rotational level of the S_0 ground state of the same spin multiplicity as the excited state. Both states are usually singlets. A non-radiative energy loss in favor of the environment takes place. The geometry of the molecule does not change during the emission so one of the higher oscillating levels of electron ground states may be filled. The fluorescence band indicates the oscillatory structure which reflects the distance of oscillatory levels of the electron ground state. It is generally shifted towards lower frequencies. This is due to radiationless (loss of energy in favor of the environment) loss of oscillatory excitation on a higher electron level before returning of the molecule to the ground state.

In flow cytometer, the time of molecule flow through the laser beam is long enough for the fluorochromes to go through the cycle of excitation-emission repeatedly, resulting in amplification of the signal emitted by the cell.

The fluorescence spectrum is shifted toward longer wavelengths with relation to the absorption spectrum, for example, the length of the excitation wave is 488nm and emission is 530nm or more. The energy difference between the absorption and emission peak is called the Stokes shift. Stokes Law (formulated in 1852) states that the wavelength of the fluorescent radiation is always bigger than the radiation wavelength exciting the fluorescence.

Argon and krypton lasers, or gas mixtures, e.g., Ar-Ne, He-Ne are often used in cytometry as a light source exciting fluorescence. Generally, lasers of different wavelengths are used- from UV through blue, green and yellow to red light (Table 1). The light emitted by the laser is coherent, monochromatic, usually horizontally polarized, of a small diameter beam, and the optimal intensity [12]. Currently, the use of diode lasers on solid is becoming more and more common.

Table 1. Examples of commercial lasers used in cytometry.

Laser		Emission lines
Argon	Ar	353-361, 488, 514 nm
Krypton-Argon	Kr-Ar	488, 568, 647 nm
Helium-Neon	He-Ne	543, 633 nm
Helium-Cadmium	He-Cd	325-441 nm

MODERN FACE OF FLOW CYTOMETRY

The use of flow cytometry to study fluorescence is currently the best method to assess the immunophenotype of individual cell populations. The fluorescence of labeled antibodies enables a quantitative and qualitative evaluation of antigens on the cell surfaces or with a intracytoplasmic expression, and therefore the presence of a specific antigen [1]. Essential is not only the information about the presence or absence of a given marker, but also data on its density on the cell surface. This type of analysis is performed by comparing the fluorescence intensity of test molecules with light intensity of microspheres coupled with known but different amounts of fluorochrome [2]. This method sets up new perspectives in medicine, especially in immunology and oncology [24]. As mentioned earlier, flow cytometry allows also to specify the physical characteristics (size, granularity), and the use of modern software allows to separate a subpopulation characterized by specific properties out of test

cells [3, 7]. The assessment of immunophenotype was of particular use in the hematooncological diagnosis. Flow cytometry is now an indispensable tool in diagnosing acute and chronic leukemia, lymphoma, myelodysplastic and myeloproliferative syndromes used alongside classical cytology, cytochemistry, cytogenetics and molecular biology [24]. Due to that it is possible to determine the origin of a given cell and the degree of its maturity and thus, diagnose the subtype of investigated leukemia. It also precisely identifies a rare antigen expression (aberrant phenotype), thereby allowing for the detection and monitoring of Minimal Residual Disease (MRD) [23]. Flow cytometry allows to detect blast cells in patients who are in an apparent complete remission [3, 11] in terms of morphological criteria. The application of this method enables the evaluation of the expression of molecules responsible for the occurrence of Multiple Drug Resistance (MDR), monitoring the effects of therapy and confirmation of disease remission [1, 3, 7]. Flow cytometry also plays an important role in the study of the mitotic cycle of tumor cells with assessment of ploidy DNA and the distribution of some proteins, like cytokines. It also enables to determine their proliferative potential and indicate the tendency to form metastases [4, 5]. Moreover, this technique also allows for the diagnosis of certain autoimmune diseases, as antiplatelet antibody test in thrombocytopenia, examination of CD95 antigen expression and apoptosis in the diagnosis of Autoimmune Lymphoproliferative Syndrome (ALPS) whereas the examination of HLA B27 antigen presence may reveal a predisposition to certain autoimmune diseases. The following diseases are also detected with the use of this method: Rheumatoid Arthritis (RA), Sjogren's Syndrome, Iritis or Autoimmune Hepatitis [3, 5]. Furthermore, the assessment of lymphocyte subpopulations in various types of immunological deficiencies, both innate and acquired, including monitoring the level of CD4 lymphocytes in patients infected with HIV, has become possible owing to flow cytometry. Not only the immunophenotype testing but also the evaluation of phagocytes function with the use of a flow cytometer allows for the diagnosis of chemotactic or phagocytic disorders, or the production of oxygen radicals in the immunological diagnostics. Flow cytometry has therefore contributed to the enormous progress into immunophenotype testing and cells involved in the body's defense mechanisms functional examinations. Moreover, transplantology is yet worth mentioning, where flow cytometry is used for the detection of Human Leukocyte Antigen (HLA) on the surface of donor and recipient cells, and to evaluate the presence of cytotoxic antibodies against the cells of the donor in the recipient's serum (crossover trial). In contrast, the control evaluation of lymphocyte subpopulation with patients after transplant provides information about the state of the immune system and the effectiveness of immunosuppressive therapy [3, 4, 5]. In comparative immunology cytometry takes part in analyzing the size and granularity of cells, and reflecting the cell cycle stage. It is also used in the assessment of morphology and platelets, and neutrophils' function. This method is also used in allergology to evaluate the effectiveness of desensitization, based on cytofluorimetric assessment of the degree of basophil degranulation or level analysis of Th1 and Th2 helper lymphocytes. The combination of flow cytometry and FISH technique in cytogenetics has become an extremely important achievement, which makes it possible to identify the chromosomal mutation, among others, the deletion of 22q11 in DiGeorge Syndrome, Philadelphia chromosome present in 95% of patients with Chronic Myeloid Leukemia. In microbiology, the cytometry allows for a rapid detection and preliminary identification of microorganisms responsible for infection. Its great advantage is the ability to analyze mixed populations which respond differently to antibacterial agents. It also enables testing the patients' immune response, detect specific antibodies and monitor the clinical status of the patient after termination of treatment. [2, 10, 19].

DIAGNOSTIC POTENTIAL OF MODERN CYTOMETERS

Sorting chromosomes, photographing each analyzed cell or simultaneous evaluation of up to 20 antigens on the same cell, provide for the exceptional abilities of cytometers [18].

One way of improving flow cytometers was to combine it with classic fluorescence microscopy. It produced one great advantage, i.e. fluorescence visualization of each analyzed cell, which has been impossible so far. This feature gives the possibility to generate two or more fluorescence signals and locate them relative to each other. What is more, except the fluorescence image of the cell those systems can also generate microscopic images [18].

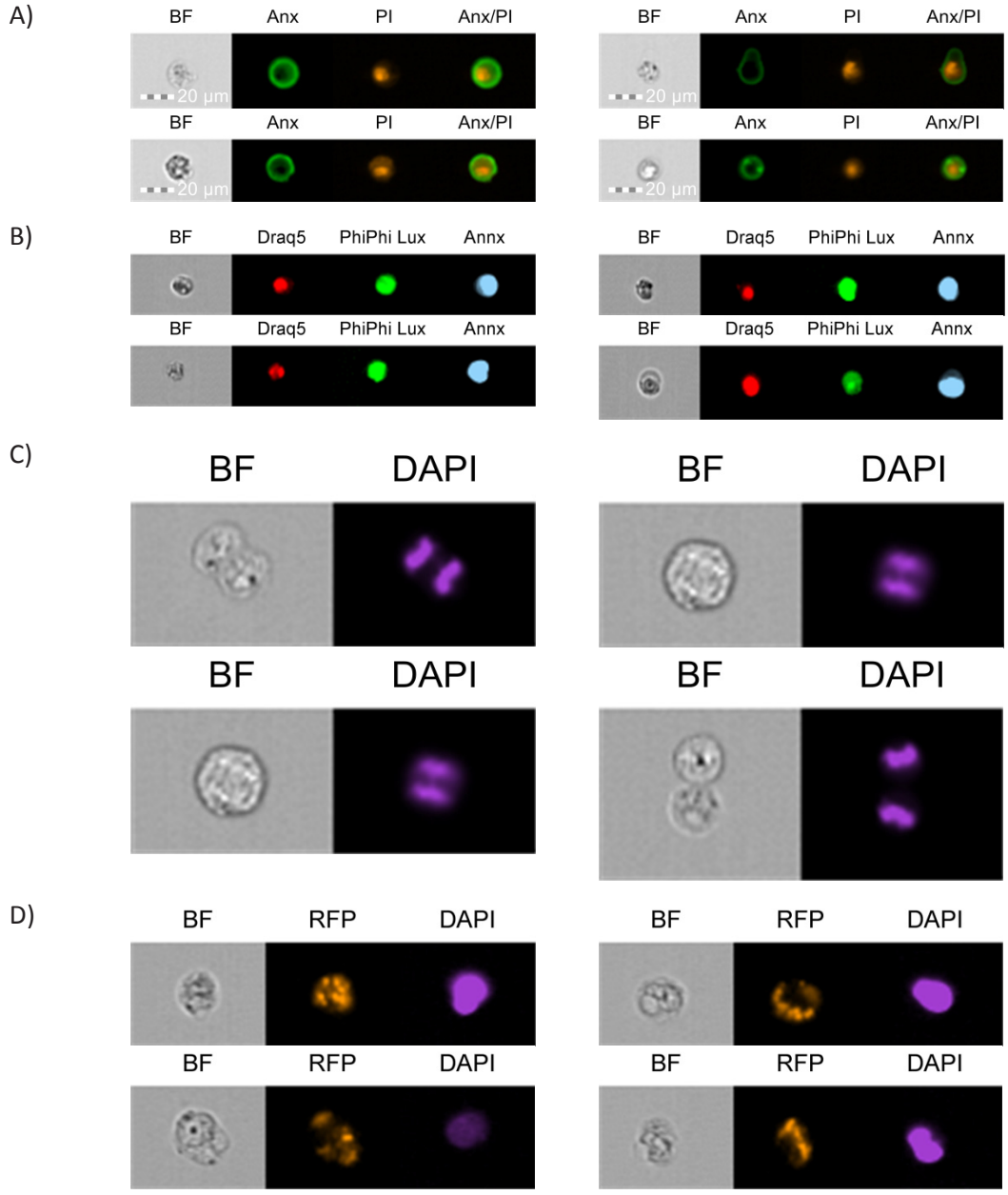

Fig. 4. Images from FlowSight cytometer (Amnis, USA) and examples of some possible applications. A) Detection of apoptosis by Annexin V-FITC and Propidium Iodide; B) Apoptosis detection by Draq5, PhiPhi Lux and Annexin V-PacificBlue; C) Cell cycle detection by DAPI; D) Starvation experiment using RFP and DAPI.

Another unusual ability of modern cytometers is cell sorting. Sorter is a flow cytometer with a function of selecting individual cell populations. Like other cytometers, it consists of three systems: hydrodynamic – provides the flow of the sample material, optical – has three lasers at most, and electric. Additionally, it is equipped with a subsystem of cell sorting which includes IntelliSort, CytoNozzle, deflection plates, Sort Chamber Door, CyCLONE, Sort Receptacle, Sort Rescue, and optionally Aerosol Evacuation Unit or Sample Cooling Kit. CytoNozzle transfers the sample to the sorting chamber, and through vibrations of a built-in piezoelectric crystal it breaks up the stream of the liquid into drops containing cells (Fig. 4). Data on flowing cells are collected during acquisition. Selection of cells starts from gating specific cell populations and defining sorting. Drops are sorted according to their deflection in the electric field produced by deflection plates. There are various sizes of Nozzle Tip (50 -200 um). Nozzle Tip should be 3 to 5 times larger than the examined cell populations. For hematopoietic system cells, optimal size is 70 µm. The entire sorting process is controlled and

stabilized by IntelliSort. The process of drop formation is affected by such environmental factors as ambient and carrier fluid temperature changes and pressure alterations. IntelliSort detects errors, interrupts sorting, and Sort Rescue prevents contamination of the already sorted samples. The pressure of the analyzed sample should be approximately 0.1 to 0.3 psi higher than that of the carrier fluid of 60 psi. Such a small pressure difference ensures laminar flow. Differential pressure of 0.5 psi and higher disturbs the flow and reduces the purity of the sorted cells, which reaches up to 99% in case of proper pressure difference. Sorting capacity reaches up to 99%. Acquisition and analysis of data is performed in Summit software run on Windows NT (Fig. 5). Currently, with the use of commercial sets of antibodies conjugated with fluorochromes, mainly hematopoietic cells are sorted including fetal erythrocytes and circulating stem cells (Fig. 6). Using a dye of affinity to DNA, Chromomycin A and Hoechst 33258 makes it also possible to perform cytometric analysis of the karyotype and sorting of individual chromosomes. Sorted cells may be used as samples for gene expression studies, and as a material for cell culture [2, 5, 15, 20-22].

Sort Logic and Statistics

Sort Decisions 3

Left 1 Stream: CD45-FITC, Purify(1)

Sort Logic	**CD45-FITC**
Limit	**None**
Sort Mode	**Purify**
Drop Envelope	**1**
Abort Stream	**Waste**
Sort Count	586077
Sort Rate	38
Accumulated Sort Count	586077
Abort Count	3789
Abort Rate	0
Accumulated Abort Count	3789
% Total	89.03%
Efficiency	99.36%

Fig. 5. Images from MoFlo Sorter (BeckmanCoulter). Summit Software – statistics on sorting cells CD45 + labelled FITC.

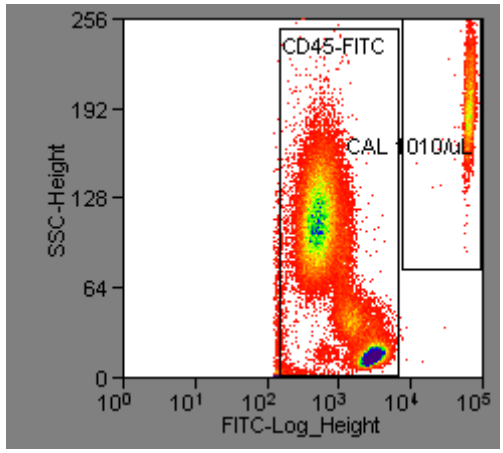

Fig. 6. Images from MoFlo Sorter (BeckmanCoulter). Cytogram presenting gated population of CD45 + labeled FITC and a calibrator for accurate determination of the number of cells analyzed in uL.

CONCLUSIONS

Recent technological advantages make flow cytometry a method used in many clinical specialties and scientific research. Its main advantage is the potential of a fast, objective, automated and multiparameter analysis and digital signal processing which contributes to more complete assessment of studied cell populations [25]. As a result, the time from disease detection to the diagnosis is unimaginably reduced. A precise determination of the disease source allows selecting the most appropriate treatment profile for a given disease. This improves the comfort of life and safety of an individual patient, and above all, has a positive effect on the health of patients, as flow cytometry is a completely non-invasive diagnostic method. Thus, such great progress will certainly contribute to wider application of flow cytometer in diagnostic and scientific research.

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petence" - the development of the Medical University of Lublin "co-financed by the European Social Fund within the Human Capital Operational Program 2007-2013

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