Received 3.30.07 | Revisions Received 4.2.07 | Accepted 4.5.07

Basic Theory and Clinical Applications of Flow Cytometry

Gabriella Henel, PhD, John L. Schmitz, PhD

(Department of Pathology and Laboratory Medicine, University of North Carolina–Chapel Hill, Chapel Hill, NC)

DOI: 10.1309/GHLEWLVOCD8025JL

Abstract

Flow cytometry (FC) has become an important component in the diagnosis and monitoring of patients with a diverse array of diseases. While the basic principals underlying FC remain relatively unchanged, the technology and array of available reagents have continued to evolve, thus expanding the list of applications in laboratory medicine. This review provides an overview of the basic theory of FC including instrumentation, sample processing, and analysis. Additionally, current and future clinical applications of FC are discussed. Among these, applications of FC in solid organ and hematopoeitic stem-cell transplantation, transfusion medicine, hematopathology, and infectious diseases are presented. Finally, applications employed primarily in clinical research are presented in anticipation of their eventual use in the clinical laboratory setting.

Flow cytometric (FC) methods for the identification and enumeration of cellular elements in various tissues have evolved into critical applications in several specialties within the clinical laboratory. These applications are used for the diagnosis, prognosis, and monitoring of patients with a variety of disease states. The ability to carry out these assays has been facilitated by continued elucidation of pathogenic mechanisms of diseases as well as advances in instrumentation and reagents for flow cytometric analysis. The role of flow cytometric analysis will continue to expand as instrumentation and reagents are continually evolving and allowing expanded applications ranging from enumeration of rare subsets of cells using polychromatic flow cytometry to assessment of the functional capacity of antigen-specific lymphocytes with specialized reagents to identify the specific responding cells.

Regardless of the evolving complexity of current applications, flow cytometric analysis relies on some basic principles, the understanding of which is important for the proper application of this technology. This overview provides a summary of the basic theory of flow cytometry and an overview of clinical applications as well as a description of newer methodologies that are likely to find their way into routine clinical application.

Basic Theory of Flow Cytometry

A flow cytometer is an instrument designed to detect and enumerate cellular elements in a suspension. The specific attributes of this technology allow for the detection and enumeration of specific cell types in complex mixtures of cells, such as whole peripheral blood. To accomplish this, flow cytometers employ the coordinated use of 3 components: the fluidics, optical, and electronic systems.[1]

The fluidics system serves to carry the cells in the sample through the cytometer for analysis. In order to do this effectively, the fluidics must generate a stream of cells that travels past an interrogation point one by one in a precise and rapid fashion. This is accomplished by generating a pressurized stream of sheath fluid into which cells are injected. The pressurized sheath fluid focuses the cells single file as they pass through the flow cell. Hydrodynamic focusing maintains the cells in single file. A uniform stream of cells is essential to ensure reproducible illumination of the cells as they intersect the laser beams of the instrument.

The attributes of cells in the sample are assessed by their ability to scatter incident laser light as well as by emission of fluorescent light if they are labeled with fluorescently-tagged reagents. Standard clinical flow cytometers contain 1 or 2 lasers that emit light of differing wavelengths. As the cells are carried through the flow cell, they reach the interrogation point at which they intersect the laser. Each cell scatters laser light that is collected along the axis of the laser beam (forward scatter) and at a 90° angle (side scatter) to the beam. Additionally, fluorescent emissions are collected at the 90° angle. Forward scatter light is collected by the forward scatter photodiode. Side scatter laser light and fluorescent emissions are directed by a series of filters and mirrors to one of several photomultiplier tubes (PMT). Instruments can be configured with specific combinations of detectors that receive defined wavelengths of light. Standard clinical flow cytometers typically have 5 or 6 PMTs, 1 for side scatter laser light and the remaining 4 or 5 for fluorescent emissions. Filter sets define the wavelength of light that reaches each PMT and, thus, the specific fluorescence that each detects (**Figure 1**). When photons of light are collected by the PMTs, they generate an electrical signal that is logarithmically

Downloaded from https://academic.oup.com/labmed/article/38/7/428/2504627 by guest on 30 November 2020

amplified and then sent to the flow cytometer's computer for storage and analysis.

The flow cytometer's computer controls the function of the instrument as well as stores data and provides software for data analysis. The signals generated by the PMTs are digitized and are proportional to the amount of scattered laser light or fluorescence detected by the PMTs. The digital signals are stored as channel numbers on one of several scales, dictated by the operator (usually 256 or 1,024 channels). For each cell that is interrogated, several parameters are generated and stored including forward and side scatter, as well as 4 or 5 fluorescent parameters. This type of data is referred to as "list mode data" and can be accessed after acquisition for analysis.

One of the unique advantages of flow cytometric analysis is the ability to employ list mode data and a process of "gating" to identify and enumerate unique subsets of cells in complex body fluids (**Figure 2**). Gating refers to the establishment of a region around a population of cells to restrict further analysis to that specific population, ignoring other cells that are present. Multiple regions can be linked together in a process of logical gating to further define specific cell types. The data that is generated can be displayed in a variety of formats but typically in single-parameter histograms or dual-parameter dot plots. The proportion of cells of interest (eg, percent positive) can be determined for each region. Enumeration (absolute counting) of cells can be achieved by using 2 different technologies, dual platform and single platform. There are significant differences between the methods used to determine absolute cell numbers. The dual-platform method relies on flow cytometric percentages of cells and hematological white blood cell (WBC) data. The single-platform method uses flow cytometers with direct absolute counting capability. There are 2 ways to determine absolute count using single-platform technology: the number of cells in a specific volume is counted, or a known number of fluorescent beads is added to tubes containing the cells.

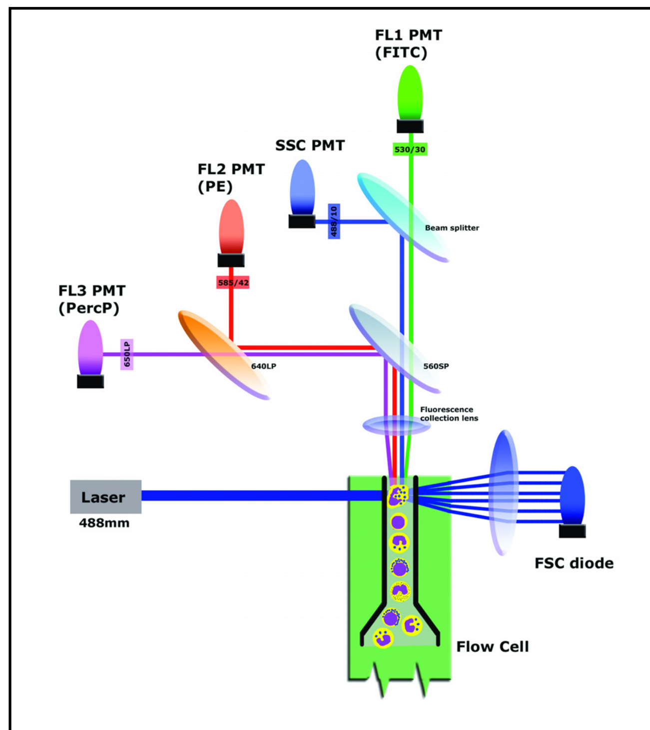

Figure 1_Structure of the flow cytometer.

Reagents for Flow Cytometric Analysis

Basic cellular characteristics, including size and internal complexity (intrinsic parameters), can be assessed via the analysis of scattered laser light. The power of flow cytometric analysis, however, is realized by the application of fluorochromes (by themselves or coupled to specific probes such as monoclonal

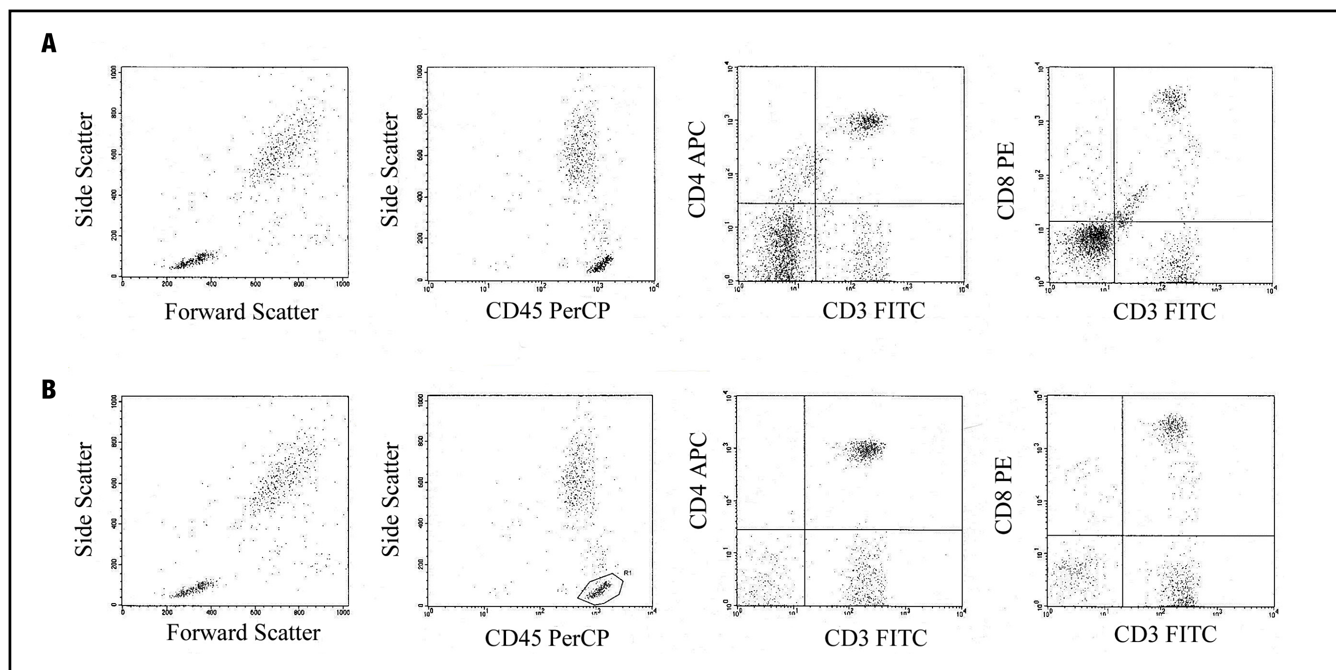

Figure 2_List mode data and a process of "gating" are employed to identify and enumerate unique subsets of cels in complex body fluids.

Downloaded from https://academic.oup.com/labmed/article/38/7/428/2504627 by guest on 30 November 2020

antibodies) to assess extrinsic parameters of cells including immunophenotype, nucleic acid content, and viability. The fluorochromes are often employed in multicolor (2, 3, or 4 fluorochromes simultaneously) analyses or in what has more recently been termed polychromatic (greater than 5 fluorochromes simultaneously) flow cytometry.

Fluorochromes are molecules that absorb light of one wavelength and emit light of higher wavelength. Emitted light is captured by the PMTs in the flow cytometer. The list of available fluorochromes for flow cytometric analysis has grown steadily over the years providing research and clinical laboratories reagents with similar excitation yet distinct emission spectra, thus allowing multicolor (polychromatic) analyses.

Nucleic acid binding fluorescent dyes are commonly-used reagents in the flow cytometry laboratory. By virtue of their ability to bind to DNA, they can be used for the quantitation of cellular DNA content. The reagents can also be used to assess the viability of a population of cells. Dyes, such as propidium iodide (PI) and 7-aminoactinomycin D (7-AAD), are excluded by viable cells, but when cell membranes are permeabilized, they allow entry of these dyes resulting in cellular fluorescence indicating cell death.

Other dyes, which bind to cytoplasmic constituents, can be employed to assess various parameters of cellular function. In the context of clinical analysis, assessing respiratory burst activity in patients suspected of having chronic granulomatous disease (CGD) represents a classic example of this approach. Dihydrorhodamine 123 (DHR123) is taken up by cells and binds to cytoplasmic proteins. In resting cells, the dye is non-fluorescent. Upon stimulation of a respiratory burst, the dye is reduced and emits a strong fluorescent signal which is readily detectable. Thus, fluorescence corresponds to an intact respiratory burst activity and rules out CGD. Other parameters such as calcium mobilization and cell division (proliferation) can be assessed with specific dyes by FC.

The most common use of fluorochromes in flow cytometric analyses involves their attachment to a monoclonal antibody and the subsequent staining of a cell preparation to identify, characterize, and enumerate a normal or abnormal population of cells. Over the years, the list of available monoclonal antibodies has grown immensely and has been accompanied by a growing list of fluorochromes for conjugation (**Table 1**). Fluorochromes, such as fluorescein (FITC) and R-phycoerythrin (PE), consist of a single molecular entity. Subsequently, the menu of available fluorochromes has been enhanced by the addition of tandem conjugates. These conjugates employ the principle of fluorescence resonance energy transfer (FRET) via the linking of an acceptor fluorochrome and an emitting fluorochrome. Incident laser light stimulates the acceptor molecule, which transfers its emitted light energy to the closely linked emitting fluorochrome. These emissions are then detected by the cytometer. More recently, the Alexa Fluor (Molecular Probes, Carlsbad, CA) reagents have been employed to further expand the list of available fluorochromes. Most recently, semiconductor nanoparticles (quantum dots) have been developed for use as fluorescent labels for flow cytometry.[2] These particles are stable and have relatively narrow emission spectra making them very useful reagents for polychromatic analysis. Along with advances in instrumentation, the expanding menu of fluorochromes has facilitated the marketing of clinical flow cytometers that can perform up to 6-color analyses. Research instruments can be configured for up to 17-color analyses.[2]

Table 1_Excitation and Emission Wavelengths of Some Commonly-Used Fluorochromes

Probe	Excitation Wavelength (nm)	Emission Wavelength (nm)
Nucleic acid probes		
DAPI (nucleic acid dye)	345	455
7-AAD (nucleic acid dye)	546	647
PI (nucleic acid dye)	536	617
Cell-function probes		
DHR	505	534
CFSE	490	518
Reactive and conjugated probes		
Fluorescein (FITC)	495	519
R-Phycoerythrin (PE)	480; 565	578
PerCP	490	675
Allophycocyanin (APC)	650	660
Cy2	489	506
Cy5	(625); 650	670
Cy5.5	675	694
Cy7	743	767
PE-Cy5 conjugates	480;565;650	670
PerCP-Cy5.5 conjugates	488;532	695
APC-Cy7 conjugates	650; 755	767
Alexa Fluors		
Alexa Fluor 350	346	445
Alexa Fluor 488	494	517
Alexa Fluor 647	650	668

Quality Control

To assure that a flow cytometer provides consistent results, optical and fluorescent standards are run daily with the results recorded in the daily quality control (QC) logbook. Commercially-available calibration beads and software are used in one approach to daily QC. The software adjusts the detectors of the flow cytometer to place the beads at target channel values and then records the detector voltage which is monitored over time. The software also measures the signal separation for each parameter and compares this value with an expected minimum separation value (sensitivity). The performance of lasers is also monitored daily. The QC log should be reviewed periodically for satisfactory performance of the instrument.

Normal controls must be tested periodically to ensure accurate sample processing and proper performance of the flow cytometer. Some tests require that a normal control is run each time the test is performed (CD4+ T-cell enumeration; detection of fetal hemoglobin). Normal controls are run periodically for other tests (immunophenotyping of leukemia and lymphoma). For test results to be acceptable, the values obtained for the normal controls should fall within the preestablished normal range. For quantitative tests, controls of at least 2 levels are run to further ensure accuracy of the testing system. These controls are often commercially available and have defined ranges.

Some assays include the staining of the cells with isotype controls. This measure allows one to establish the level of nonspecific antibody binding. Most monoclonal antibodies are IgG1 or IgG2a isotypes. Fluorochromes conjugated to IgG1 and IgG2a are therefore the most often used for isotype control. Alternatively, a negatively-staining population within the gated region may be used to assess nonspecific binding, thus eliminating the need for additional tubes of isotype control-stained cells. However, different cell types may have different autofluorescent properties, which must be considered when using this approach.

Downloaded from https://academic.oup.com/labmed/article/38/7/428/2504627 by guest on 30 November 2020

Flow Cytometry in the Diagnosis of Immunodeficiency Disorders

Enumeration of Lymphocyte Subsets

Flow cytometry with monoclonal antibodies is a critical component of the evaluation of patients with primary or secondary immunodeficiencies.[3] Three types of FC analyses are common for the evaluation of patients with possible immunodeficiencies: enumeration of peripheral blood cells, detection of function-associated cell surface or intracellular antigens, and assessment of functional attributes of cells.

Abnormalities in the number of circulating lymphocyte subsets are seen in many different primary immunodeficiencies.[4] The enumeration of lymphocyte subsets is thus important in the diagnostic approach for patients with these deficiencies. The most noted secondary immunodeficiency, HIV infection, promoted the widespread use of the clinical application of lymphocyte subset enumeration. After infection, patients exhibit a gradual decline in the CD4 T-lymphocyte count, typically over a period of years, until they develop the manifestations of advancing immunodeficiency (ie, AIDS) and, subsequently, death. Enumeration of the CD4 T-lymphocyte count has several roles in HIV infection. In a symptomatic patient, the CD4 count can contribute to the diagnosis. The CD4 count is an important prognostic factor for HIV-infected patients, helping to gauge the time to AIDS or death. In addition, the CD4 count helps guide decisions regarding the institution of antiretroviral drugs as well as prophylaxis for opportunistic infections.[5] The CD4 count is monitored longitudinally every 3 to 6 months in untreated patients and every 2 to 4 months in treated patients.[5-7] When a patient is placed on antiretroviral drugs, the CD4 count usually, but not always, increases. A CD4 decrease while on therapy may indicate the development of resistance to one or more of the antiretroviral drugs being taken by the patient.

The need for reliable and reproducible CD4 counts was a major driving force in the development of clinical FC stimulating developments in instrumentation and flourochromes as well as a greater understanding of the complexity of lymphocyte subsets. More recently, the need for simplified and less-costly methods for CD4 T-cell enumeration has stimulated development of alternatives to standard FC. Alternatives include smaller instruments using single-platform technology that are dedicated to CD4 counting approaches that employ a limited number of antibodies and alternate gating strategies to reduce costs (panleucogating, single CD4).[8]

Evaluation of Neutrophil Function

The analysis of neutrophil function in patients suspected of having CGD is a useful example of assessment of the functional capacity of a cell by FC. Chronic granulomatous disease is a primary immunodeficiency resulting from defects in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system in neutrophils. In normal functioning neutrophils, the NADPH-oxidase participates in the generation of H_2O_2 and superoxide anion which contribute to the killing of ingested microbes. Defects in the subunits of the oxidase system lead to a failed or reduced respiratory burst and defective killing. As described above, DHR123 can be used to assess this response.[9]

Phagocytosis is the engulfing and ingestion of bacteria or other foreign bodies by cells. Defects in phagocytosis are rare; however, FC assays have been developed to assess this immune function. Typically, these assays employ a fluorescently-labeled inert particle or whole bacterial cell which is added to a suspension of white cells. Uptake of the particles or cells is assessed by identification of white cells exhibiting fluorescence of the ingested particles. An important challenge of assessing phagocytosis by FC is to differentiate bound particles from internalized particles;[10,11] however, a commercially available kit that can exclude those particles from the analysis that are bound, but not phagocytosed, is available (Vybrant Phagocytosis Assay Kit; Invitrogen, Carlsbad, CA). This kit uses fluorescein isothiocyanate (FITC)-labeled, preopsonized *Escherichia coli* as targets for the phagocytes. Briefly, fluorescently-labeled *E. coli* is added to whole blood, and after a short incubation time at 37°C the reaction is stopped and the percentage of FITC-positive cells is determined by FC. Monoclonal antibodies to CD14 or CD33 are used to identify monocytes and granulocytes.

Assessing T-cell Proliferation

To successfully fight off an infection, lymphocytes need to proliferate exponentially after the initial exposure to their cognate antigen. For the analysis of lymphocyte proliferation by FC, the fluorescent dye succinimidyl ester of carboxyfluorescein diacetate [5(6)-CFSE] is used. The CFSE dye permanently binds to both intracellular and cell surface proteins by reacting with lysine side chains and other available amine groups.

The fluorescence intensity of CFSE can be measured by FC. Upon cell division, CFSE is distributed equally between the daughter cells, and the mean fluorescence intensity (MFI) of the new generation of cells is therefore half of the MFI of the parent cells. Each halving of CFSE intensity represents a cell division.[12] The number of divisions, which can be followed, is limited only by the level of auto fluorescence of the unlabeled cells, and the uniformity in size of the labeled cell population.

Assessing Specific Antigens

Several primary immunodeficiencies involve the absence or reduced expression of a cell surface or intracellular molecule that can be assessed by FC.

Diagnosis of Hyper-Immunoglobulin M Syndrome by Flow Cytometry

Hyper-immunoglobulin M (HIGM) syndrome is a primary combined immunodeficiency caused by mutations in the gene encoding the CD40 ligand (CD154).[13] Hyper-IgM syndrome is characterized by normal or elevated serum IgM levels with absent or reduced serum levels of the other immunoglobulin isotypes.[14]

A flow cytometric test is now available in clinical laboratories to detect abnormalities in CD40 ligand expression.[15,16] On normal T cells the expression of CD40 ligand is significantly upregulated within 4 hours after stimulation; however, if there is a mutation in the CD40L gene, T cells cannot induce the expression of CD40L on their cell surface. The absence of CD40L on stimulated T cells is therefore an indicator of HIGM syndrome. This assay has been routinely used to diagnose HIGM syndrome, to monitor functional immune reconstitution following bone marrow transplant in patients with HIGM syndrome,[17] and to measure CD40L expression in children with HIV infection.[18] Furthermore, women who are carriers of the X-linked hyper-IgM syndrome (XHIM) can be identified since they have 2 distinct populations of stimulated T cells: 1 that expresses CD40L and 1 that does not.

Downloaded from https://academic.oup.com/labmed/article/38/7/428/2504627 by guest on 30 November 2020

Flow Cytometric Determination of Leukocyte Adhesion Deficiency

The ability of leukocytes to leave the blood stream and enter tissues is essential for their proper function; they need to accumulate rapidly at the sites of inflammation or tissue injury. Adhesion molecules, expressed by leukocytes and vascular endothelial cells, help mediate leukocyte migration. The important adhesion molecules in the leukocyte-vascular endothelium interaction belong to 3 families: selectins, integrins, and some proteins belonging to the immunoglobulin superfamily. Selectins are lectins that bind to carbohydrate ligands expressed on the surface of endothelial cells. There are 3 different selectins: L-selectins are expressed on leukocytes, and P- and E-selectins are expressed by endothelial cells. Integrins are a family of heterodimeric proteins consisting of noncovalently-linked α and β chains. LFA-1 and Mac-1 are two leukocyte integrins that are essential for leukocyte migration. CD18 is the β chain common to both LFA-1 (CD11aCD18) and Mac-1 (CD11bCD18).

Defects in adhesion molecules involved in leukocyte migration prevent neutrophils and macrophages from reaching the sites of infection. This results in widespread pyogenic bacterial infections.

Three types of leukocyte adhesion deficiency (LAD) have been described: LAD-I, LAD-II, and LAD-III. LAD-I is the most common of the 3. It is an autosomal recessive syndrome caused by various mutations in the CD18 molecule. More than 40 mutations have been identified in LAD-I.[19] The severity of the immunodeficiency is directly related to the degree of CD18 deficiency. Patients with less than 2 percent of the normal cell surface expression of CD18 have a more severe form of the disease, with earlier, more frequent, and more serious infections that often lead to death in infancy. Patients with CD18 surface expression between 2% and 30% of normal levels manifest a milder form of immunodeficiency, with fewer serious bacterial infections and often survival into adulthood.

LAD-II is a rare, autosomal recessive syndrome that is due to the absence of the sialyl-Lewis X (CD15s), a carbohydrate moiety that is necessary for the interaction of selectins expressed on neutrophils with selectins expressed on endothelial cells, and therefore for the rolling of neutrophils before extravasation.[20]

LAD-III is a very rare, autosomal recessive disorder that has only been described in a handful of cases. In those cases, the structure and expression level of integrins were normal; however, there was a defect in their activation.[21]

There are simple tests available in clinical FC laboratories to measure cell-surface expression of leukocyte adhesion molecules. A combination of monoclonal antibodies to the molecules CD18, CD11b, CD15, and CD11c are used to screen patients for possible LAD-I and LAD-II.

Cytokine Flow Cytometry

To measure antigen-specific T-cell responses, several methods can be used (proliferation, cytokine production, and cytotoxicity); however, these methods are cumbersome and difficult to use for enumeration of antigen-specific T cells. The main advantage of cytokine flow cytometry (CFC) is that it can relatively easily quantify the percentage of antigen-specific T cells and determine their phenotypic characteristics. Cytokine flow cytometry can also be used for assessing the activity of other cell types, such as natural killer (NK) cells, monocytes, and dendritic cells.

The cells of interest are stimulated for 6 hours with a specific antigen either in whole blood or in peripheral blood mononuclear cells (PBMC) in the presence of a secretion inhibitor such as brefeldin A (BFA). The cells are then fixed and permeabilized so the flourophore-labeled anti-cytokine antibodies can enter the cell and bind to their target. Staining for subset-specific cell surface molecules can be done at the same time as the intracellular staining or before the fixation step. The cells are acquired, the cell population of interest is gated, and the results are reported as the percent of cytokine-positive cells (**Figure 3**). Recently, to make CFC a more high-throughput assay, it has been optimized for multiwell format.[22]

Applications in Hematology

Paroxysmal Nocturnal Hemoglobinuria Testing

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematologic disorder characterized by complement-mediated intravascular hemolysis. Paroxysmal nocturnal hemoglobinuria develops as the result of the clonal expansion of the hematopoietic stem cells with mutations in the X-linked PIG-A gene.[23]

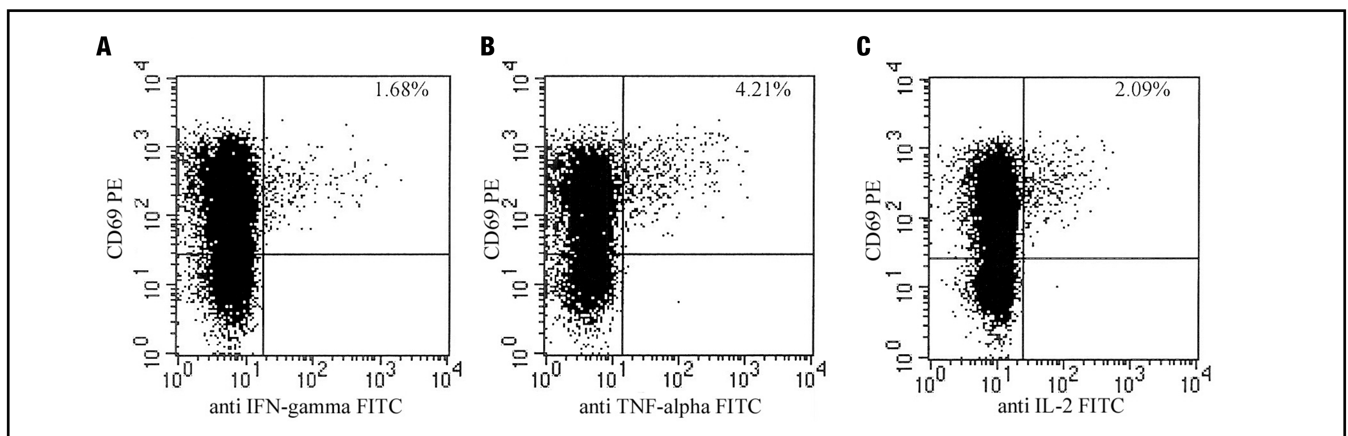

Figure 3_Cells are acquired, the population of interest is gated, and results are reported as the percent of cytokine-positive cells.

Downloaded from https://academic.oup.com/labmed/article/38/7/428/2504627 by guest on 30 November 2020

This gene is essential for the biosynthesis of the glycosyl-phosphatidyl-inositol (GPI) anchor, and mutations in the PIG-A gene results in a deficiency or lack of all GPI-anchored proteins on the cell surface.[24] Such GPI-anchored proteins include the complement regulatory proteins CD55 and CD59. Paroxysmal nocturnal hemoglobinuria red blood cells lack the cell surface expression of these regulatory proteins and therefore are susceptible to complement-mediated hemolysis. The Ham's acid hemolysis test and the sugar water test have historically been used in the diagnosis of PNH.[25] However, FC is the current gold standard for laboratory detection of PNH clones, since the absence of GPI-anchored proteins can be assessed by staining for the presence of cell surface CD55 and CD59 on red blood cells (RBCs) or neutrophils.[26] This assay is both more sensitive and more specific than the other tests employed for PNH testing. By using FC tests, one can identify different types of PNH cells. Type I cells have normal levels of GPI-anchored protein expression; type II cells have intermediate levels; and type III cells completely lack expression of these proteins.[27]

Immunophenotyping of Leukemia and Lymphoma

One of the major applications of FC in today's clinical laboratories is the phenotypic characterization of hematologic malignancies, the various types of leukemias and lymphomas.[28] In the pursuit of reaching a definitive diagnosis, various laboratory studies, such as immunohistochemical immunophenotyping, cytomorphological, histological, cytogenetic, and molecular studies, are performed. In many instances, FC studies are also used to reach a definitive diagnosis since many of the hematologic malignancies have unique immunophenotypic profiles that can be characterized by FC.

Hematologic malignancies arise as the result of clonal expansion of abnormal lymphoid or myeloid cells blocked at different stages of cell maturation. Normal hematopoietic cells express a variety of cell surface molecules during their lifespan; therefore, each developmental stage can be characterized by the pattern of cell surface molecules that are expressed. The detection of hematologic malignancies by FC relies on the principle that malignant cells express cell surface molecules in different patterns than normal cells. There are several patterns of abnormal antigen expression: expression of an antigen at the wrong stage of development; expression of an non-lineage specific antigen; abnormal increase or decrease in expression levels (or a complete lack of expression) of normal antigens; and uniform, homogenous, expression of antigens by a cell population that normally displays more diverse expression (light chain restriction).

In most clinical laboratories, panels of monoclonal antibodies to lineage-specific (eg, lymphoid or myeloid) and maturation-specific antigens are used to identify cell populations with abnormal antigen expression patterns. Flow cytometric immunophenotyping has many advantages such as weakly-expressed surface antigens may be detected; multicolor analysis allows for an accurate description of the antigen profile of specific cells; 2 simultaneous hematologic malignancies may be detected within the same tissue site; and a wide variety of specimens are suitable for analysis (peripheral blood, bone-marrow aspirates, core biopsies, fine-needle aspirates, and fresh-tissue biopsies).

Flow cytometry can also be used for the evaluation of therapeutic responses and for the detection of minimal residual disease (MRD) after therapy. Minimal residual disease monitoring relies on the determination of the major antigen signature of the malignant cell at diagnosis, which is applied to post-treatment samples to detect the presence of the malignant clone.[29,30]

Applications in Transplantation

Monitoring Patients Receiving Anti-Rejection Therapy

Recipients of solid organ transplants can develop cellular or humoral rejection that, in certain cases, requires strong immunosuppressive therapy for adequate treatment. Among the agents used are antibody-based therapies such as OKT3, thymoglobulin, and rituximab.[31,32] These mono- and polyclonal preparations deplete T and B lymphocytes, thus interfering with the alloresponse. Some patients develop antibodies to these agents that interfere with their action. As such, patients may be monitored for depletion of T or B cells to ensure that the agents are depleting these cell types. Flow cytometry with T and B cell markers is employed to enumerate these cells types.

Detection of HLA Alloantibody

Candidates for solid-organ transplants are screened for the presence of preformed HLA alloantibody, which they may have developed from previous blood transfusions, transplants, or pregnancies.[33] Cytotoxicity-based methods have been the standard for detection and characterization of the alloantibodies. More recently, enzyme-linked immunosorbent assay (ELISA)- and FC-based methods have been developed.[34,35] Flow-based methods have been shown to provide the most sensitive approach for detection of HLA alloantibody.[36] In addition, with the advent of beads sensitized with purified HLA antigens, flow-based methods have evolved into highly-specific approaches that eliminate detection of cell-reactive but non-HLA-directed antibodies. A variety of reagents are available to qualitatively screen for the presence of HLA antibody or to determine the specificity (target HLA antigen) of the alloantibody.

Prior to transplant, the absence of specific donor-directed antibody is verified by the performance of a donor crossmatch. Like antibody screening, crossmatching has employed a variety of techniques. Initially, cytotoxicity-based methods were the sole approach used; however, during the last 20 years, FC crossmatching has evolved into a critical component of the crossmatch repertoire. As for antibody screening, the main advantage of FC-based crossmatching is its greater sensitivity than the cytotoxicity-based method. The clinical importance of this increased sensitivity has been validated in many studies that have documented improved transplant outcomes in flow-crossmatch-negative versus flow-crossmatch-positive transplants.[37]

Enumeration of CD34+ Stem and Progenitor Cells

Enumeration of CD34-expressing stem and progenitor cells has emerged as a common assay in stem cell transplantation. As hematopoietic stem cells mature in the bone marrow, they express a variety of cell surface proteins in a coordinated fashion. One of the earliest appearing markers on the cell surface is the CD34 antigen.[38] As cells mature, they lose expression of

Downloaded from https://academic.oup.com/labmed/article/38/7/428/2504627 by guest on 30 November 2020

this antigen on the cell surface; thus, the presence of CD34 is a marker of stem and progenitor cells. Enumerating the number of CD34-bearing cells provides an estimate of the repopulating capacity of a stem and progenitor cell product.[39,40] The ability to enumerate CD34-bearing cells has proven advantageous over the previously-employed approach of determining the total mononuclear cell dose as the guide for the quantity of stem cell preparation to infuse.[41]

A straightforward immunophenotyping panel can be used to determine the proportion and absolute number of CD34-bearing cells in the total white cell population (CD45 positive) of bone marrow or peripheral blood stem cell preparations. These numbers are then used to determine the amount of stem cell product required for infusion.

Applications in Transfusion Medicine

Detection of Fetal Hemoglobin

The detection and enumeration of fetal-hemoglobin-containing RBCs is one of the newer flow cytometric applications.[42] Detection of fetal hemoglobin has several clinical applications: detection and quantification of fetomaternal hemorrhage (FMH)n either in the case of trauma with suspected placental injury or in cases of Rh incompatibility;[43] quantification of hemoglobin F in adult RBCs in various hemoglobinopathies; monitoring the effect of fetal hemoglobin-stimulating agents on the hemoglobin F levels in patients with sickle cell disease and thalassemias;[44] and monitoring the effectiveness of intrauterine transfusions (IUT).[45] The most commonly-used application, however, is to detect FMH. If the normal physiological barrier between fetal and maternal circulation is disrupted, FMH occurs. Trauma, especially abdominal trauma, to the pregnant woman can cause FMH.[46]

Hemolytic disease of the newborn can occur when the mother is Rh-negative and the fetus is Rh-positive. It can be prevented by administering the right amount of anti-Rh Ig to the mother during pregnancy.[46] The correct dose is calculated after the frequency of fetal-hemoglobin-containing cells in the mother's peripheral blood is determined.[47]

Since hemoglobin is an intracellular molecule, the RBCs must be permeabilized first in order for the hemoglobin F-specific monoclonal antibody to bind to its target.

Measuring Residual White Cells in Leukocyte-Reduced Blood

The presence of leukocytes in blood and platelet products is associated with adverse transfusion reactions which include alloimmunization of RBC antigens, platelet antigens, and HLA and other leukocyte-associated antigens;[48] viral and bacterial infections;[49,50] and transfusion-associated lung injury (TRALI).[51]

Leukocyte reduction of blood product to a threshold of 5 × 10^6 WBC/unit has been shown to reduce the risks of adverse transfusion reactions. Flow cytometry is commonly used for residual leukocyte enumeration. The commercially-available LeucoCount Kit (BD Biosciences, San Jose, CA) utilizes propidium iodine (PI), a fluorescent dye that binds to nucleic acid, to stain the DNA of nucleated cells. Since RBCs and platelets do not have nuclei, they will not be stained. The absolute count of residual WBCs is determined by adding a known number of beads to the blood product (ie, single platform technology).

Future Directions

In clinical laboratories, FC is a powerful diagnostic tool with new tests continually being developed. Examples are described below.

Polychromatic Flow Cytometry

In the pursuit to reach a definitive diagnosis (especially in the case of hematological malignancies), the characterization of the expression pattern of many proteins, cell surface and cytoplasmic, is desirable. It is advantageous to know which proteins are coexpressed on certain subpopulations of cells. In many instances the small volume of a sample obtained from the patient is a limiting factor for the number of tests that can be performed. Therefore, there is a need for the development of polychromatic flow cytometric tests. Multiparameter FC (up to 17 colors) has been available in research settings for some time now,[52,53] but now flow cytometers are available for clinical testing that can detect up to 6 fluorochromes. It is safe to say that soon clinical laboratories will be equipped with instruments that can detect even more fluorochromes.[54] This will reduce the requirement for large volumes of specimen, the time needed for sample processing, and the total number of antibodies needed for analysis. Furthermore, this technique will enable laboratory professionals to detect and identify rare subsets of cells.

Applications in Diagnosis of Allergy

The most commonly-used test today for the detection of functional allergic response is the skin test; however, the identification of the allergen causing the allergic reaction is not always straightforward. The use of FC to assess the reaction of basophils to allergens might provide a solution.[55] The basis of this assay is simple. CD63 molecules in resting basophils are located in the membranes of the intracellular granules and are not detectable on the cell surface. If basophils are sensitized in vivo with allergen-specific IgE, then exposure to the allergen in vitro will result in their degranulation, and therefore the expression of CD63 on the cell surface of the activated basophils will increase in less than 10 minutes.[56] The expression level of another molecule, CD203c, will also increase.[57] Monoclonal antibodies to CD63 and CD203c can therefore be used to determine the effect of specific allergens on the basophils. An increase in their cell-surface expression level indicates an allergic reaction.

Antifungal Susceptibility Testing

The clinical use of FC is ever expanding as more and more clinical fields are taking advantage of this technique. One such area is clinical microbiology. Antifungal susceptibility testing (AST) can now be done using FC.[58] One advantage of flow cytometric AST over the conventional methods is the 4-hour incubation time compared with current methods that require an incubation time of 24 to 48 hours or more. After incubation with the antifungal agent, the fungal cells are stained with a specific fluorescent dye and the changes in fluorescence intensity of stained cells due to effects of the antifungal agents is measured by FC. For instance, fungal cells might become more fluorescent if they are susceptible to the antifungal agent.

Downloaded from https://academic.oup.com/labmed/article/38/7/428/2504627 by guest on 30 November 2020

1. Shapiro HM. *Practical Flow Cytometry*, 4th ed. Hoboken, NJ: Wiley-Liss; 2003.
2. Chattopadhyay PK, Price DA, Harper TF, et al. Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry. *Nat Med*. 2006;12:972–977.
3. Folds JD, Schmitz JL. Clinical and laboratory assessment of immunity. *J Allergy Clin Immunol*. 2003;111:S702–S711.
4. Notarangelo L, Casanova JL, Conley ME, et al. Primary immunodeficiency diseases: An update from the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee Meeting in Budapest, 2005. *J Allergy Clin Immunol*. 2006;117:883–896.
5. Yeni PG, Hammer SM, Carpenter CC, et al. Antiretroviral treatment for adult HIV infection in 2002: Updated recommendations of the International AIDS Society—USA Panel. *JAMA*. 2002;288:222–235.
6. Haubrich RH, Currier JS, Forthal DN, et al. A randomized study of the utility of human immunodeficiency virus RNA measurement for the management of antiretroviral therapy. *Clin Infect Dis*. 2001;33:1060–1068.
7. Dybul M, Fauci AS, Bartlett JG, et al. Guidelines for using antiretroviral agents among HIV-infected adults and adolescents. *Ann Intern Med*. 2002;137:381–433.
8. Glencross D, Scott LE, Jani IV, et al. CD45-assisted PanLeucogating for accurate, cost-effective dual-platform CD4+ T-cell enumeration. *Cytometry*. 2002;50:69–77.
9. O'Gorman MR, Corrochano V. Rapid whole-blood flow cytometry assay for diagnosis of chronic granulomatous disease. *Clin Diagn Lab Immunol*. 1995;2:227–232.
10. Heinzelmann M, Gardner SA, Mercer-Jones M, et al. Quantification of phagocytosis in human neutrophils by flow cytometry. *Microbiol Immunol*. 1999;43:505–512.
11. White-Owen C, Alexander JW, Sramkoski RM, et al. Rapid whole-blood microassay using flow cytometry for measuring neutrophil phagocytosis. *J Clin Microbiol*. 1992;30:2071–2076.
12. Lyons AB. Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. *J Immunol Methods*. 2000;243:147–154.
13. Conley ME, Notarangelo LD, Etzioni A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). *Clin Immunol*. 1999;93:190–197.
14. Winkelstein JA, Marino MC, Ochs H, et al. The X-linked hyper-IgM syndrome: Clinical and immunologic features of 79 patients. *Medicine* (Baltimore). 2003;82:373–384.
15. Freyer DR, Gowans LK, Warzynski M, et al. Flow cytometric diagnosis of X-linked hyper-IgM syndrome: Application of an accurate and convenient procedure. *J Pediatr Hematol Oncol*. 2004;26:363–370.
16. O'Gorman MR, Zaas D, Paniagua M, et al. Development of a rapid whole blood flow cytometry procedure for the diagnosis of X-linked hyper-IgM syndrome patients and carriers. *Clin Immunol Immunopathol*. 1997;85:172–181.
17. Jacobsohn DA, Emerick KM, Scholl P, et al. Nonmyeloablative hematopoietic stem cell transplant for X-linked hyper-immunoglobulin M syndrome with cholangiopathy. *Pediatrics*. 2004;113:122–127.
18. O'Gorman MR, DuChateau B, Paniagua M, et al. Abnormal CD40 ligand (CD154) expression in human immunodeficiency virus-infected children. *Clin Diagn Lab Immunol*. 2001;8:1104–1109.
19. Roos D, Meischl C, de Boer M, et al. Genetic analysis of patients with leukocyte adhesion deficiency: Genomic sequencing reveals otherwise undetectable mutations. *Exp Hematol*. 2002;30:252–261.
20. Etzioni A, Tonetti M. Leukocyte adhesion deficiency II-from A to almost Z. *Immunol Rev*. 2000;178:138–147.
21. Alon R, Etzioni A. LAD-III, a novel group of leukocyte integrin activation deficiencies. *Trends Immunol*. 2003;24:561–566.
22. Maecker HT, Rinfret A, D'Souza P, et al. Standardization of cytokine flow cytometry assays. *BMC Immunol*. 2005;6:13.
23. Bessler M, Mason PJ, Hillmen P, et al. Paroxysmal nocturnal haemoglobinuria (PNH) is caused by somatic mutations in the PIG-A gene. *Embo J*. 1994;13:110–117.
24. Boccuni P, Del Vecchio L, Di Noto R, et al. Glycosyl phosphatidylinositol (GPI)-anchored molecules and the pathogenesis of paroxysmal nocturnal hemoglobinuria. *Crit Rev Oncol Hematol*. 2000;33:25–43.
25. Hartmann RC, Jenkins DE, Jr., Arnold AB. Diagnostic specificity of sucrose hemolysis test for paroxysmal nocturnal hemoglobinuria. *Blood*. 1970;35:462–475.
26. Hall SE, Rosse WF. The use of monoclonal antibodies and flow cytometry in the diagnosis of paroxysmal nocturnal hemoglobinuria. *Blood*. 1996;87:5332–5340.
27. Rosse WF. Paroxysmal nocturnal hemoglobinuria as a molecular disease. *Medicine* (Baltimore). 1997;76:63–93.
28. Kaleem Z, Crawford E, Pathan MH, et al. Flow cytometric analysis of acute leukemias. Diagnostic utility and critical analysis of data. *Arch Pathol Lab Med*. 2003;127:42–48.
29. Campana D, Coustan-Smith E. Minimal residual disease studies by flow cytometry in acute leukemia. *Acta Haematol*. 2004;112:8–15.
30. Zwick D, Cooley L, Hetherington M. Minimal residual disease testing of acute leukemia by flow cytometry immunophenotyping: A retrospective comparison of detection rates with flow cytometry DNA ploidy or FISH-based methods. *Lab Hematol*. 2006;12:75–81.
31. Koch A, Daniel V, Dengler TJ, et al. Effectivity of a T-cell-adapted induction therapy with anti-thymocyte globulin (Sangstat). *J Heart Lung Transplant*. 2005;24:708–713.
32. Vieira CA, Agarwal A, Book BK, et al. Rituximab for reduction of anti-HLA antibodies in patients awaiting renal transplantation: 1. Safety, pharmacodynamics, and pharmacokinetics. *Transplantation*. 2004;77:542–548.
33. Goodman RS, Taylor CJ, O'Rourke CM, et al. Utility of HLAMatchmaker and single-antigen HLA-antibody detection beads for identification of acceptable mismatches in highly sensitized patients awaiting kidney transplantation. *Transplantation*. 2006;81:1331–1336.
34. Pei R, Lee JH, Shih NJ, et al. Single human leukocyte antigen flow cytometry beads for accurate identification of human leukocyte antigen antibody specificities. *Transplantation*. 2003;75:43–49.
35. Worthington JE, Robson AJ, Sheldon S, et al. A comparison of enzyme-linked immunoabsorbent assays and flow cytometry techniques for the detection of HLA specific antibodies. *Hum Immunol*. 2001;62:1178–1184.
36. Hoy T GS, Shenton BK, Bell AE, et al. *Further Clinical Applications*, 3rd ed. New York: Oxford University Press; 2000.
37. Karuppan SS, Ohlman S, Moller E. The occurrence of cytotoxic and non-complement-fixing antibodies in the crossmatch serum of patients with early acute rejection episodes. *Transplantation*. 1992;54:839–844.
38. Krause DS, Fackler MJ, Civin CI, et al. CD34: Structure, biology, and clinical utility. *Blood*. 1996;87:1–13.
39. Douay L, Gorin NC, Mary JY, et al. Recovery of CFU-GM from cryopreserved marrow and in vivo evaluation after autologous bone marrow transplantation are predictive of engraftment. *Exp Hematol*. 1986;14:358–365.
40. To LB, Haylock DN, Simmons PJ, et al. The biology and clinical uses of blood stem cells. *Blood*. 1997;89:2233–2258.
41. Bensinger WI, Longin K, Appelbaum F, et al. Peripheral blood stem cells (PBSCs) collected after recombinant granulocyte colony stimulating factor (rhG-CSF): An analysis of factors correlating with the tempo of engraftment after transplantation. *Br J Haematol*. 1994;87:825–831.
42. Brown M, Wittwer C. Flow cytometry: Principles and clinical applications in hematology. *Clin Chem*. 2000;46:1221–1229.
43. The estimation of fetomaternal haemorrhage. BCSH Blood Transfusion and Haematology Task Forces. *Transfus Med*. 1999;9:87–92.
44. Mundee Y, Bigelow NC, Davis BH, et al. Flow cytometric method for simultaneous assay of foetal haemoglobin containing red cells, reticulocytes and foetal haemoglobin containing reticulocytes. *Clin Lab Haematol*. 2001;23:149–154.
45. Wynn R, Dixon S, al-Ismail SA, et al. Flow cytometric determination of pre-transfusion red cell volume in fetuses and neonates requiring transfusion based on RhD+ dilution by transfused D- red cells. *Br J Haematol*. 1995;89:620–622.
46. Dziegiel MH, Nielsen LK, Berkowicz A. Detecting fetomaternal hemorrhage by flow cytometry. *Curr Opin Hematol*. 2006;13:490–495.
47. Hartwell EA. Use of Rh immune globulin: ASCP practice parameter. *Am J Clin Pathol*. 1998;110:281–292.
48. Vamvakas EC. Meta-analyses of studies of the diagnostic accuracy of laboratory tests: A review of the concepts and methods. *Arch Pathol Lab Med*. 1998;122:675–686.
49. Andreu G, Morel P, Forestier F, et al. Hemovigilance network in France: Organization and analysis of immediate transfusion incident reports from 1994 to 1998. *Transfusion*. 2002;42:1356–1364.
50. Blajchman MA, Goldman M, Baeza F. Improving the bacteriological safety of platelet transfusions. *Transfus Med Rev*. 2004;18:11–24.

Downloaded from https://academic.oup.com/labmed/article/38/7/428/2504627 by guest on 30 November 2020

51. Blajchman MA. The clinical benefits of the leukoreduction of blood products. *J Trauma*. 2006;60:S83–90.
52. Perfetto SP, Chattopadhyay PK, Roederer M. Seventeen-colour flow cytometry: Unravelling the immune system. *Nat Rev Immunol*. 2004;4:648–655.
53. Petrausch U, Haley D, Miller W, et al. Polychromatic flow cytometry: A rapid method for the reduction and analysis of complex multiparameter data. *Cytometry A*. 2006;69:1162–1173.
54. Wood B. 9-color and 10-color flow cytometry in the clinical laboratory. *Arch Pathol Lab Med*. 2006;130:680–690.
55. Ebo DG, Sainte-Laudy J, Bridts CH, et al. Flow-assisted allergy diagnosis: Current applications and future perspectives. *Allergy*. 2006;61:1028–1039.
56. Paris-Kohler A, Demoly P, Persi L, et al. In vitro diagnosis of cypress pollen allergy by using cytofluorimetric analysis of basophils (Basotest). *J Allergy Clin Immunol*. 2000;105:339–345.
57. Buhring HJ, Streble A, Valent P. The basophil-specific ectoenzyme E-NPP3 (CD203c) as a marker for cell activation and allergy diagnosis. *Int Arch Allergy Immunol*. 2004;133:317–329.
58. Vale-Silva LA, Buchta V. Antifungal susceptibility testing by flow cytometry: Is it the future? *Mycoses*. 2006;49:261–273.

Downloaded from https://academic.oup.com/labmed/article/38/7/428/2504627 by guest on 30 November 2020