Review Article

Application of Flow Cytometry in Transfusion Medicine

Rajesh B Sawant

Department of Transfusion Medicine, Histocompatibility and Immunogenetics, Kokilaben Dhirubhai Ambani Hospital, Mumbai, Maharashtra, India

ABSTRACT

Flow cytometry-based testing approaches have no longer remained restricted to the research laboratories. With widening of its horizons in the field of diagnostics in oncology and transplantation medicine, the flow cytometer-based testing approach is now relevant to many aspects of the field of transfusion medicine. Two of the most important applications of flow cytometry in transfusion medicine are the enumeration of CD34-positive cells in the peripheral blood and stem cell product and the enumeration of residual white blood cell counts in blood product as a quality control measure. The flow cytometer-based crossmatch for detection of donor-specific anti-human leukocyte antigen antibodies is also gaining popularity in the field of histocompatibility and immunogenetics. The utility of this platform in the field of immunohematology is also increasing and looks promising for clinical decision-making in the near future.

KEYWORDS: *CD34-positive cells, flow cytometry, human leukocyte antigen crossmatch, residual white blood cell counts, transfusion medicine*

Received: March, 2019.
Accepted: March, 2019.

INTRODUCTION

A PubMed search (including MEDLINE) was done using keywords: flow cytometry, transfusion medicine, CD34-positive cells, residual white blood cell (rWBC) counts, and human leukocyte antigen (HLA) crossmatch. The aspects in which the author has first-hand experience have been included in this review. Till the 1990s, flow cytometers were used only in research laboratories. In the current era, they have become portable, affordable, and hence, widely acceptable in various fields of the laboratory. In transfusion medicine, the major applications of flow cytometry are in the field of hematopoietic stem cell transplantation (HSCT), study of blood group antigens, detection of fetomaternal hemorrhage (FMH), and enumeration of rWBC counts in cellular blood components. The flow cytometer has now become an important diagnostic tool which complements the results of serology and genetic testing.

The first clinical flow cytometer was used in the 1970s. It worked on the principle of measurement of different characteristics of single cells (or particles) by streaming them in a "sheath" fluid across a source of laser light. The scattering of light (forward scatter and side scatter) aids in the recognition of cells of different granularity and size. When cells labeled with fluorochrome-conjugated antibodies pass through the laser, the fluorochrome is excited and emits light of a different wavelength. The light is sent to different detectors known as photomultiplier tubes (PMTs) by the use of optical filters and reflectors. The electrical pulses originating from the light signal detected by the PMTs are then processed and each event can be plotted on a graphical scale, and the plots or histograms can be used to visualize the results.

FACTORS INFLUENCING THE USE OF FLOW CYTOMETER FOR COUNTING OF CELLS

Preanalytic factors – The most significant effect is that of pipetting techniques, centrifugation, reagent storage, erythrocyte-lysing procedure, and washing. Apart from these, the specificity, quality, and quantity of the antibodies as well as the type of fluorescent dye used also have an effect on the performance of the flow cytometric counts.

Analytic factors such as gating strategies, measuring principles, and statistical aspects also play a major role in accurate test performance.

Access this article online

Quick Response Code:

Website: www.gjtmonline.com

DOI: 10.4103/GJTM.GJTM_26_19

Address for correspondence:
Dr. Rajesh B Sawant, E-mail: rajesh.b.sawant@relianceada.com, rbshil@gmail.com

How to cite this article: Sawant RB. Application of flow cytometry in transfusion medicine. Glob J Transfus Med 2019;4:16-20.

Flow Cytometry in Transfusion Medicine: Major Applications

i. HSCT and solid organ transplantation
   a. Determination and quantification of hematopoietic progenitor cells in
      - Bone marrow
      - Peripheral blood (PB)
      - Cord blood.
   b. HLA crossmatching
   c. Quantification of leukocyte subsets.
ii. Adoptive immunotherapy and further therapeutic applications
   a. Analysis of minimal residual disease
      - Analysis of viability/apoptosis after manipulation/cryopreservation of cells
      - Analysis of dendritic cells
      - Analysis of specificity and functionality of T-cells
      - Analysis of natural killer (NK) cell phenotype and cytotoxicity.
iii. Quality control of blood components
   a. Quantification of residual leukocytes in leukoreduced blood components
   b. Functional investigations of cells or cell fractions in blood components (viability, apoptosis, etc.)
   c. Contamination of blood components (e.g., with tumor cells or bacteria).
iv. Immunohematologic diagnostics
   a. Red blood cell (RBC) integrity and immunology
      - Detection and quantification of antibodies bound to RBCs
      - Detection and quantification of antigens on RBCs
      - Detection and quantification of mixed populations of RBCs (chimerism and FMH)
      - Genetic disorders of erythrocytes (paroxysmal nocturnal hemoglobinuria).
   b. Platelet integrity and immunology
      - Detection and quantification of antibodies against platelets
      - Detection and quantification of platelet antigens
      - Platelet crossmatching
      - Determination of platelet function.
   c. Granulocyte/monocyte integrity and immunology
      - Detection of granulocyte function and antigens
      - Detection of phagocytosis.

CD34-Positive Cell Enumeration

Failure to harvest the desired number of CD34+ cells results in emotional disappointment for the patient and a financial burden on the hospital. Therefore, a clearly defined cutoff value for circulating CD34+ cells which can be used as a criterion to initiate leukapheresis and also to predict the yield is highly desirable.

Flow cytometry is widely used in the HSCT setting. The most important analysis is to define the content of CD34-positive hematopoietic progenitor cells in stem cell grafts[1] and to measure the T-cell content prior to allogeneic transplantations as per the guidelines issued by the Joint Accreditation Committee of international society for cell and gene therapy (ISCT) and European society for blood and marrow transplantation (EBMT). For operational and economical efficiency, it would be beneficial if sufficient PB progenitor cells could be obtained from the fewest collections, and the optimal timing of the leukapheresis could be reliably predicted. For obtaining a yield of $>1 \times 10^6$/Kg CD34+ cells in a single apheresis procedure, our study recommends a PB-CD34+ cell count $>20 \times 10^3/\mu l$.[2]

Applications of flow cytometry in bone marrow transplantation also include hematopoietic stem cell graft manipulation in the pretransplantation phase by determination of the efficacy of *ex vivo* T-cell graft depletion. In the posttransplantation phase, it can assist in the evaluation of immune recovery, graft rejection, graft-versus-host disease (GVHD), and graft-versus-leukemia effect.[3,4]

Morris *et al.* found that donor T-cells confer protection on transplant recipients by promoting bone marrow engraftment, providing immunity against opportunistic infections, and eliminating residual malignant cells resistant to the host-conditioning regimen.[5]

Quality Control of Blood Components

Leukocytes are in general not meant for transfusions but are invariably present as contaminants in all blood components, and they mediate adverse reactions such as febrile nonhemolytic transfusion reactions (FNHTRs),[6,7] alloimmunization,[8,9] and transmission of infectious diseases (e.g., cytomegalovirus [CMV]),[10] GVHD, transfusion-related acute lung injury, and immunomodulation. Exclusive use of leukodepleted components can decrease alloimmunization, CMV transmission, and FNHTRs. Improvement in the extent of WBC reduction has been achievable in RBCs and platelets due to recent advances in filtration as well as apheresis techniques so that components with $<5 \times 10^6$ contaminating WBCs can be readily available.

The new generation of leukoreduction filters can achieve removal of up to 99.99% of leukocytes from

cellular blood components such as RBCs and platelet concentrates.

Quality assurance of the leukofiltration process includes product specification, process control, and validation of the rWBC enumeration methodology.

An essential element of leukoreduction process control is the availability of counting assays for residual leukocytes that have good performance characteristics. Automated counting methods such as those based on flow cytometry are less subjective and provide greater accuracy and precision than do manual methods such as the Nageotte hemocytometer counting methods.[11] Leukodepleted blood components are expected to contain 1 × 10^6–5 × 10^6 WBCs per unit. Techniques for enumerating rWBCs include the cell counters which can be used for preleukodepletion samples (least count: 100 WBCs/μl), microscopic counting methods – Nageotte chamber (least count: 10 WBCs/μl), molecular-based enumeration (least count: 1 WBCs/μl), and flow cytometry (least count: 0.1 WBCs/μl). Quality assurance of leukocyte depletion processes is a demanding issue. Modern and quick validation tools are required for rWBC enumeration. Even thawed plasma may contain enough viable lymphocytes to induce harm to the recipient.[12] Blood bags from different manufacturers are used by every center for blood collection. The type of filter used for filtration also affects the outcome of leukodepletion. The Nageotte chamber method has a low accuracy (largely underestimation of WBCs), especially with RBC components. As low as 0.08 × 10^6 WBCs/L in RBCs and 0.25 × 10^6 WBCs/L in PCs can be detected with the flow cytometry platform.[13] It is necessary to prepare and run samples within 48 h of leukodepletion for the best results. Although the components are labeled as leukodepleted (rWBC count <5 × 10^6), a lot of variation may occur in the rWBC count and this is of great clinical significance for certain patient subgroups. Achieving a good intra- and interlaboratory variation in the rWBC enumeration is the goal today. Having an external quality assessment program for rWBC enumeration and standardization of the manual Nageotte chamber counting is the need of the hour today to ensure safer transfusions.

Discrepancies have been reported between counting methods based on the Nageotte hemocytometer and flow cytometry. Discrepancies historically have been attributed to differences in sensitivity and reproducibility between flow cytometry and manual counting methods. Janatpour *et al*.[14] have extensively studied flow cytometry-based counting methods, such as Leucocount™, and have suggested valuable clues to understand the flow cytograms for rWBC count that must be visually inspected before reporting results. These cytograms provide additional information not always readily apparent with other automated systems. Flow cytograms provide additional insight into counting discrepancies. For example, a flow cytometer might produce a higher leukocyte count than a Nageotte method because it counts large fragments and bare nuclei that are eliminated from some Nageotte counting criteria. Conversely, debris might be erroneously enumerated as leukocytes by flow cytometry unless the cytogram is visually inspected. This information may be useful for laboratories that are converting from a manual Nageotte hemocytometer method to an automated flow cytometry-based method such as Leucocount™ or vice versa.

Three types of atypical flow cytogram patterns were observed during process validation or routine quality control of leukoreduced RBC components.

a. Fixation artifact: Fixation of control or test samples can alter the staining intensity compared with fresh cells
b. "Rain" pattern: Flow cytometry methods count slightly damaged leukocytes not removed during leukoreduction. Slightly damaged leukocytes appear on a flow cytogram like "rain" falling from a well-defined "cloud" of intact residual leukocytes. Discrepancies between automated flow cytometry results and subjective manual counting methods can occur.
c. Autofluorescence-debris pattern: Cell debris and age-related changes in the sample can cause shifts in the fluorescence staining pattern, resulting in erroneous test results.

Review of flow cytograms is essential for accurate reporting of flow cytometry-based methods for enumerating residual leukocytes in leukoreduced blood components.

Detection of Anti-Human Leukocyte Antigen Antibodies in the Solid Organ Transplant Setting

The flow cytometer platform is now used routinely by some centers for detection of donor-specific antibodies prior to solid organ transplantation. This test is supposed to be very sensitive than the traditional complement-dependent cytotoxicity assay used for crossmatching the donor samples with the recipient.

This method detects low levels of antibodies, but even the presence of nonspecific antibodies leads to a positive crossmatch result. The results also depend on the quality of lymphocytes available for the crossmatch. Therefore,

this test is used in combination with other testing methods for clinical decision-making.

Flow Cytometry for Immune Monitoring

Flow cytometry can now play a significant role for the standard immune monitoring by measuring the numbers of T, B, and NK cells in patients who are maintained on immunosuppressive therapy after receiving a solid organ transplant. Among patients who are on immunosuppressive drugs, many authors have highlighted the significant correlation between the incidence of opportunistic infections and the levels of the immune regulatory cells.[15,16]

Analysis of red blood cell antigens

Serology at times leads to ambiguous and confusing results while testing for various blood group antigens including the "D" antigen and various new blood group systems.[17,18]

Flow cytometry is commonly used to evaluate cases of "D" variants by analysis of the antigen expression and antigen site density.

Flow cytometry has also been used for the analysis of the RBC storage lesion and determination of the expression of ABO blood group and RhD (ABH) antigens[19] and also to study alteration of ABH antigen levels in leukemia patients.

Other important applications include analysis of subgroups of A[20] and B blood group and the Bombay and Para-Bombay blood group.

Testing for Chimerism

Chimerism is a state when two or more different cell populations are evident during serology testing. This can happen due to recent transfusion, post transplantation, or due to certain clonal disorders. Chimerism analysis by flow cytometry has been extensively reviewed by Bluth *et al*.[21]

Fetomaternal Hemorrhage

It is very important to determine the extent of FMH in the clinical setting of a RhD-negative female mother carrying a RhD-positive fetus. The extent of FMH is calculated to administer an adequate dose of RhD immunoglobulin to the mother for the prevention of hemolytic disease of the newborn (HDN). Several groups have proposed the use of anti-fetal hemoglobin (HbF) for the identification of fetal cells in different settings, used by itself or in combination with anti-D.

Anti-HbF is also used in combination with an antibody toward carbonic anhydrase (CA), an enzyme that is fully expressed only after birth; hence, all cells positive for CA are derived from the mother, and maternal cells containing HbF can be distinguished from fetal cells.[22] The prevention of HDN by monitoring the FMH levels has been successfully achieved by flow cytometric analysis.

Flow Cytometry in Testing of Human Platelet Antigens

Anti-human platelet antigens-1a (HPA-1a) is clinically a very important platelet antibody and is implicated in around 80% of cases with fetal and neonatal alloimmune thrombocytopenia in Caucasians.[23] In a Caucasian population, the expected frequency to be HPA-1a negative is approximately 2%. Keeping a registry of platelet donors negative for HPA-1a has been a successful strategy implemented by referral transfusion centers for ensuring prompt availability of the appropriate platelet transfusion support for cases of alloimmune thrombocytopenia in the newborns. This screening is efficiently performed on the flow cytometry platform. Apart from the above, the flow cytometer has also been used for platelet crossmatching and the analysis of platelet storage lesions. CD62P (P-selectin) is probably the most frequently used marker for determination of storage lesion in platelet units.

Flow Cytometry in Direct Antiglobulin Test-Negative Autoimmune Hemolytic Anemia

Chaudhary *et al*.[24] have studied the application of flow cytometry in the detection of RBC-bound autoantibodies in direct antiglobulin test-negative autoimmune hemolytic anemia (AIHA). They have concluded that flow cytometry is a very useful tool in assessing Coombs-negative AIHA and should be employed when conventional tube technique and column agglutination technology give discordant results, and there is a strong clinical suspicion of AIHA.

Flow cytometry has thus gained popularity now not only in the basic research laboratories but also in the routine laboratory medicine including applications in transfusion medicine and transplantation medicine. In combination with other diagnostic tools, this technology provides valuable clinical information. Reproducibility of the test results remains the greatest challenge today to further enhance the value of this valuable tool in the clinical setting.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Whitby A, Whitby L, Fletcher M, Reilly JT, Sutherland DR, Keeney M, *et al.* ISHAGE protocol: Are we doing it correctly? Cytometry B Clin Cytom 2012;82:9-17.
2. Sawant RB, Rajadhyaksha SB. Correlation of CD34+cell yield in peripheral blood progenitor cell product with the pre-leukapheresis cell counts in peripheral blood. J Assoc Physicians India 2005;53:1031-5.
3. Storek J, Joseph A, Espino G, Dawson MA, Douek DC, Sullivan KM, *et al.* Immunity of patients surviving 20 to 30 years after allogeneic or syngeneic bone marrow transplantation. Blood 2001;98:3505-12.
4. Bordin JO, Heddle NM, Blajchman MA. Biologic effects of leukocytes present in transfused cellular blood products. Blood 1994;84:1703-21.
5. Uhlmann EJ, Isgriggs E, Wallhermfechtel M, Goodnough LT. Prestorage universal WBC reduction of RBC units does not affect the incidence of transfusion reactions. Transfusion 2001;41:997-1000.
6. Kishino K, Muroi K, Kawano C, Obata T, Sugano N, Nakagi Y, *et al.* Evaluation of engraftment by ABO genotypic analysis of erythroid burst-forming units after bone marrow transplantation. Leuk Res 2002;26:13-7.
7. Morris ES, Macdonald KP, Hill GR. Stem cell mobilization with G-CSF analogs: A rational approach to separate GVHD and GVL? Blood 2006;107:3430-5.
8. Williamson LM, Wimperis JZ, Williamson P, Copplestone JA, Gooi HC, Morgenstern GR, *et al.* Bedside filtration of blood products in the prevention of HLA alloimmunization – A prospective randomized study. Alloimmunisation study group. Blood 1994;83:3028-35.
9. Trial to Reduce Alloimmunization to Platelets Study Group. Leukocyte reduction and ultraviolet B irradiation of platelets to prevent alloimmunization and refractoriness to platelet transfusions. N Engl J Med 1997;337:1861-9.
10. Bowden RA, Slichter SJ, Sayers M, Weisdorf D, Cays M, Schoch G, *et al.* A comparison of filtered leukocyte-reduced and cytomegalovirus (CMV) seronegative blood products for the prevention of transfusion-associated CMV infection after marrow transplant. Blood 1995;86:3598-603.
11. Dzik S, Moroff G, Dumont L. A multicenter study evaluating three methods for counting residual WBCs in WBC-reduced blood components: Nageotte hemocytometry, flow cytometry, and microfluorometry. Transfusion 2000;40:513-20.
12. Hiruma K, Okuyama Y. Effect of leucocyte reduction on the potential alloimmunogenicity of leucocytes in fresh-frozen plasma products. Vox Sang 2001;80:51-6.
13. Dumont LJ, Dzik WH, Rebulla P, Brandwein H. Practical guidelines for process validation and process control of white cell-reduced blood components: Report of the biomedical excellence for safer transfusion (BEST) working party of the international society of blood transfusion (ISBT). Transfusion 1996;36:11-20.
14. Janatpour K, Paglieroni TG, Schuller L, Foley K, Rizzardo T, Holland PV. Interpretation of atypical patterns encountered when using a flow cytometry-based method to detect residual leukocytes in leukoreduced red blood cell components. Cytometry 2002;50:254-60.
15. Calarota SA, Zelini P, De Silvestri A, Chiesa A, Comolli G, Sarchi E, *et al.* Kinetics of T-lymphocyte subsets and posttransplant opportunistic infections in heart and kidney transplant recipients. Transplantation 2012;93:112-9.
16. Kho MM, Bouvy AP, Cadogan M, Kraaijeveld R, Baan CC, Weimar W. The effect of low and ultra-low dosages thymoglobulin on peripheral T, B and NK cells in kidney transplant recipients. Transpl Immunol 2012;26:186-90.
17. Svensson L, Hult AK, Stamps R, Ångström J, Teneberg S, Storry JR, *et al.* Forssman expression on human erythrocytes: Biochemical and genetic evidence of a new histo-blood group system. Blood 2013;121:1459-68.
18. Danger Y, Danard S, Gringoire V, Peyrard T, Riou P, Semana G, *et al.* Characterization of a new human monoclonal antibody directed against the vel antigen. Vox Sang 2016;110:172-8.
19. Fibach E, Sharon R. Changes in ABH antigen expression on red cells during *in vivo* aging: A flow cytometric analysis. Transfusion 1994;34:328-32.
20. Berneman ZN, van Bockstaele DR, Uyttenbroeck WM, Van Zaelen C, Cole-Dergent J, Muylle L, *et al.* Flow-cytometric analysis of erythrocytic blood group A antigen density profile. Vox Sang 1991;61:265-74.
21. Bluth MH, Reid ME, Manny N. Chimerism in the immunohematology laboratory in the molecular biology era. Transfus Med Rev 2007;21:134-46.
22. Porra V, Bernaud J, Gueret P, Bricca P, Rigal D, Follea G, *et al.* Identification and quantification of fetal red blood cells in maternal blood by a dual-color flow cytometric method: Evaluation of the fetal cell count kit. Transfusion 2007;47:1281-9.
23. Kamphuis MM, Paridaans N, Porcelijn L, De Haas M, Van Der Schoot CE, Brand A, *et al.* Screening in pregnancy for fetal or neonatal alloimmune thrombocytopenia: Systematic review. BJOG 2010;117:1335-43.
24. Chaudhary R, Das SS, Gupta R, Khetan D. Application of flow cytometry in detection of red-cell-bound IgG in coombs-negative AIHA. Hematology 2006;11:295-300.