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Application of flow cytometry in transfusion medicine: The Sanjay Gandhi Post Graduate Institute of Medical Sciences, India experience

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Abstract:

The application of flow cytometry (FC) is diverse and this powerful tool in used in multiple disciplines such as molecular biology, immunology, cancer biology, virology, and infectious disease screening. FC analyzes a single cell or a particle very rapidly as they flow past single or multiple lasers while suspended in buffered solution. FC has a great impact in the field of transfusion medicine (TM) due to its ability to analyze individual cell population and cell epitopes by sensitive, reproducible, and objective methodologies. The main uses of FC in TM are detection of fetomaternal hemorrhage, diagnosis of paroxysmal nocturnal hemoglobinuria, quantification of D antigen, detection of platelet antibody, quality control of blood components, for example, residual leukocyte counts and evaluation of CD34-positive hematopoietic progenitor cells in stem cell grafts. In recent years, FC has been implemented as an alternative method for the detection and characterization of red cell autoantibodies in autoimmune hemolytic anemia. Many workers considered FC as a very good complement when aberrant expression of various erythrocyte antigens needs to be elucidated. It has been extensively used in the resolution of ABO discrepancies and chimerism study. FC has also been used successfully in various platelet immunological studies. In the recent past, FC has been used in several studies to assess the platelet storage lesions and elucidate granulocyte/monocyte integrity and immunology. FC analysis of CD34+ stem cells is now the method of choice to determine the dosage of the collected progenitor cells. The technique is vastly used to evaluate residual leukocytes in leukodepleted blood components. We conclude that flow cytometers are becoming smaller, cheaper, and more user-friendly and are available in many routine laboratories. FC represents a highly innovative technique for many common diagnostic and scientific fields in TM. Finally, it is the tool of choice to develop and optimize new cellular and immunotherapeutic trials.

Keywords:

Coomb's negative autoimmune hemolytic anemia, fetomaternal hemorrhage, flow cytometry, platelet antibody, transfusion medicine

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Introduction

The application of flow cytometry (FC) is diverse and this powerful tool in used in multiple disciplines today. Important disciplines include molecular biology, immunology, cancer biology, virology, and infectious disease screening. FC which primarily consists of the three systems, namely fluidics, optics, and electronics is an advanced technology that analyzes a single cell or a particle very rapidly as they flow past single or multiple lasers while suspended in buffered solution [Figure 1].[1-3] Each particle is analyzed for visible light scatter and one or multiple fluorescence parameters. Visible light scatter is measured in two different directions, the forward direction or the Forward Scatter which

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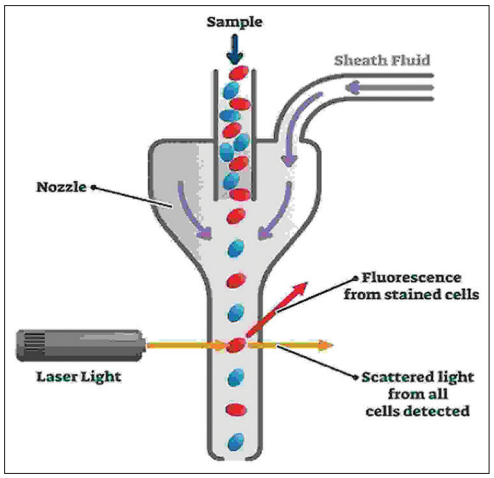

Figure 1: A schematic diagram of a flow cytometer

measures the relative size of the cell and at 90° or the side scatter which indicates the internal complexity or granularity of the cell. Importantly, the samples under investigation are prepared for fluorescence measurement through transfection and expression of fluorescent proteins, staining with fluorescent dyes, or staining with fluorescently conjugated antibodies.[2-5] Importantly, the FC allows simultaneous characterization of mixed populations of cells from blood and bone marrow as well as solid tissues that can be dissociated into single cells such as lymph nodes, spleen, mucosal tissues, and solid tumors. In addition to the analysis of populations of cells, a major application of FC is sorting cells for further analysis. The data in FC are analyzed conventionally using the two-parameter histogram or dot plot gating; however with the advancement in instrumentation advanced data analysis tools are applied and explored today.[4-7]

Flow Cytometry in Transfusion Medicine

Today transfusion medicine (TM) is a multidisciplinary specialty with various recent developments that continue to challenge immunohematologists. Definitely, FC has a great impact in the field of TM due to its ability to analyze individual cell population and cell epitopes by sensitive, reproducible, and objective methodologies.[8]

Through the years FC has been used to examine red blood cells (RBC) in different immunohematology settings; although it has never completely found its way into the routine immunohematology laboratories. Today, the main use of FC analysis in TM is for the diagnosis of the direct antiglobulin test (DAT) negative autoimmune hemolytic anemia (AIHA), detection of fetomaternal hemorrhage (FMH), diagnosis of paroxysmal nocturnal hemoglobinuria (PNH), quantification of D antigen, detection of platelet antibody, quality control of blood components, for example, residual leukocyte counts, elucidating aberrant antigen expression, and red cell phenotyping.[9,10]

As cellular therapies are now a part of TM in many places, therefore, FC is widely used in the hematopoietic stem cell transplantation setting for analyzing the content of CD34 positive hematopoietic progenitor cells in stem cell grafts and to measure T-cell content before allogeneic transplantations.[9-11]

The Department of TM at the Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Lucknow, India, is an academic facility with a fully-fledged immunohematology laboratory. The laboratory handles numerous requests and samples for FC for the diagnosis of DAT-negative AIHA and PNH; the detection of FMH and platelet antibody and quantification of D antigen. Few of these FC applications performed in the immunohematology laboratory are discussed below.

Flow cytometry in the diagnosis of direct antiglobulin test negative autoimmune hemolytic anemia

In recent years, FC has been implemented as an alternative method for the detection and characterization of red cell autoantibodies in AIHA. Garraty and Arndt cited FC to be a highly sensitive antibody detection technique with the ability to detect as low as 30–40 molecules per RBC.[12] Chaudhary *et al*. from SGPGIMS, India investigated that AIHA patients with negative DAT results by conventional tube technique or column agglutination techniques (CAT) can be easily diagnosed using FC. Authors commented that FC is a very useful tool in assessing "Coomb's negative AIHA" and should be employed when other DAT methods give discordant results and there is a strong clinical suspicion of AIHA. However to rule out false-positive results in FC due to its extreme sensitivity proper positive and negative controls have to be executed during running test samples in FC for diagnosis of AIHA [Figure 2].[13] In another study, FC was used to confirm the result of CAT in the determination of immunoglobulin G (IgG) subclass and titer. The authors concluded that the advantage of FC allows it to diagnose the so-called "Coomb's negative" AIHA which at times are missed by the CAT technique as it requires approximately 200 molecules per red cells for a positive DAT.[14]

Flow cytometry in fetomaternal hemorrhage

An adequate dose calculation of anti-D Ig in any sensitized mother is possible through the estimation of correct FMH volume. In this regard, the Kleihauer Betke test or acid elution test is the most commonly used one, but it is time-consuming, difficult and not easily amenable to standardization.[15,16] These factors led many immunohematology laboratories to adopt

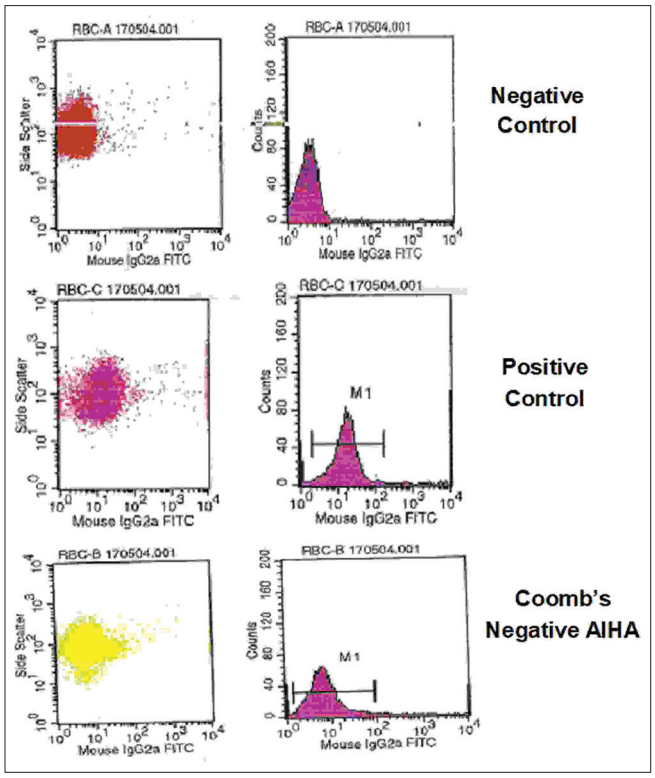

Figure 2: Flow cytometry in the diagnosis of Coomb's negative autoimmune hemolytic anemia

the new methods of FMH quantifications of which FC has been designated as the most sensitive, reproducible, and rapid technique.[17-19] Two FC techniques such as the indirect immunofluorescence technique and direct immunofluorscence technique are advocated to determine FMH. While the former technique is based on the labeling of RhD antigen with an anti-D reagent followed by the addition of preconjugated IgG antibody as described previously; the later uses fluorescein isothiocyanate labeled anti-CD45 monoclonal antibody and phycoerythrin (PE) labeled anti-fetal haemoglobin (HbF) antibody to detect HbF.[15] Agarwal *et al*. from SGPGIMS, India, found both techniques sensitive overall sample dilutions tested. They observed FC as the most sensitive technique that could detect RhD-positive/HbF-positive events accurately even at a cell concentration of 0.06% which at times other techniques fail [Figure 3].[20] It was Nance *et al*. in 1989 who first reported FC as a simple technique to quantitate FMH.[21] Almost all authors who worked on FC and FMH found the technique readily adaptable to laboratories with a high degree of accuracy and reproducibility.[17,22] Agarwal *et al*. also concluded that detection of FMH volume is an important step in the management of maternal RhD iso-immunization, therefore, each laboratory should adopt technique, which is simple, rapid, sensitive, reproducible, and importantly economically affordable.[20]

Flow cytometry in paroxysmal nocturnal hemoglobinuria

PNH is an acquired clonal stem cell disorder, resulting in intravascular hemolysis, cytopenia of variable degree, and thrombotic events. The hallmark of PNH blood cells is a deficiency or absence of proteins that utilize the glycosylphosphatidylinositol (GPI) anchor for their attachment to the plasma membrane. The absence of certain GPI-anchored complement regulatory proteins, such as CD55 and CD59, accounts for the complement-mediated hemolysis that characterizes PNH. FC can detect cells with absent or reduced expression of GPI-anchored proteins on cell surfaces. FC immunophenotyping can discriminate between cell populations with differential expression or the absence of one or more GPI-anchored proteins on erythrocytes, leukocytes, and platelets. In most cases, the technique uses PE-conjugated monoclonal antibodies (MoAb), namely CD55 and CD59 [Figure 4].[23-25]

Gupta *et al*. from SGPGIMS compared four techniques for the diagnosis of PNH and investigated that small PNH clones (≤1%), differential expression of GPI-anchored proteins, and defective leukocytes could be easily identified by FC using MoAb to GPI-anchored proteins [Figure 4].[23] Other workers on PNH also commented that the use of sensitive techniques like FC enables the detection of a small PNH clone, which does not produce symptoms or signs of hemolysis, in a variety of clonal hematological disorders, such as aplastic anemia, myelodysplasia, myeloproliferative, and lymphoproliferative disorders as well as in normals.[24-26] Kashyap *et al*. also concluded that PNH is not a rare disorder in the Indian subcontinents; however, a high index of clinical suspicions and evaluation of erythrocytes, granulocytes, and monocyte by FC is necessary for its detection to understand the PNH pathophysiology more closely.[27]

Flow cytometry in the quantification of D antigen

FC is considered as a very good complement when aberrant expression of various erythrocyte antigens needs to be elucidated. The use of FC for the analysis of protein/glycoprotein in blood group antigens/systems such as RH and KEL has been discussed.[28-31]

Genes of the Rhesus (Rh) blood group system, namely *RHD* and *RHCE* encode for the D, C, c, and E, e antigens, of which D antigen is the most immunogenic, followed by c and E. It has been observed that as low as 0.1–1 ml of D-positive red cells can induce anti-D formation in D-negative recipients. This may cause severe transfusion reactions and hemolytic disease of the fetus and newborn.[32] The strength of antigenic determinants present on the surface of red cells is one of the factors which determine the immunogenicity of an antigen.[33]

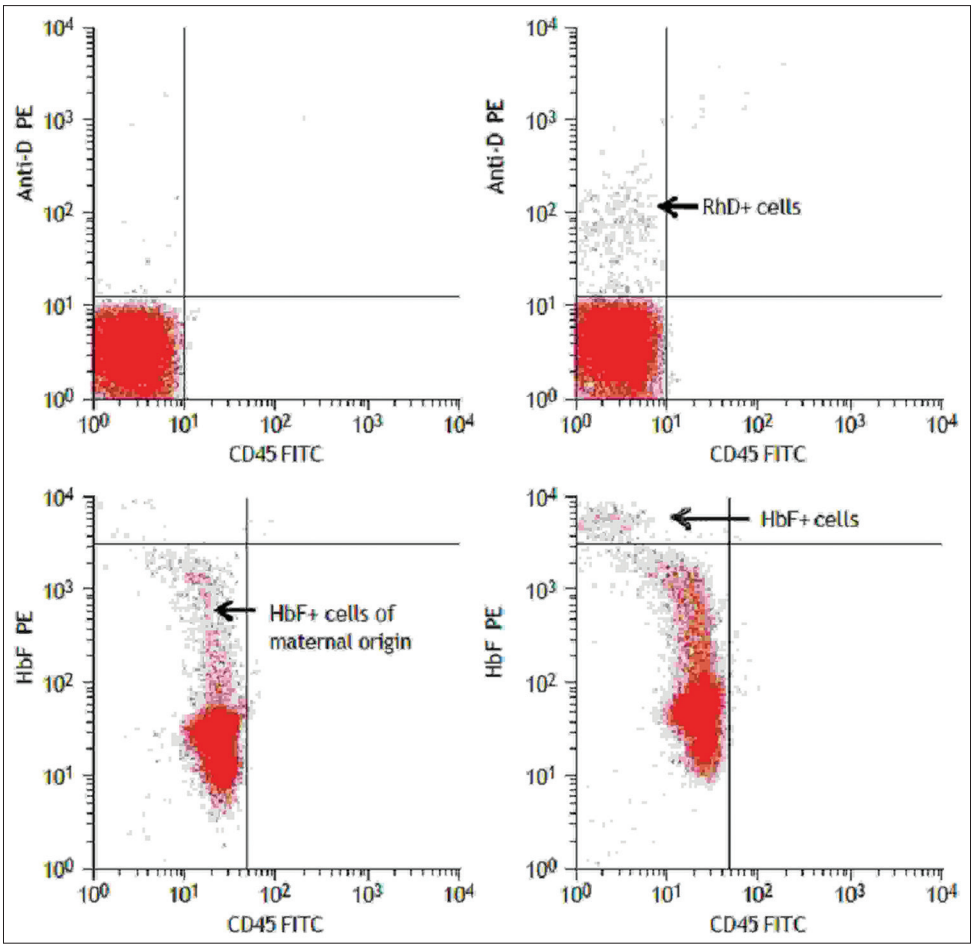

Figure 3: Flow cytometric analysis of fetomaternal hemorrhage

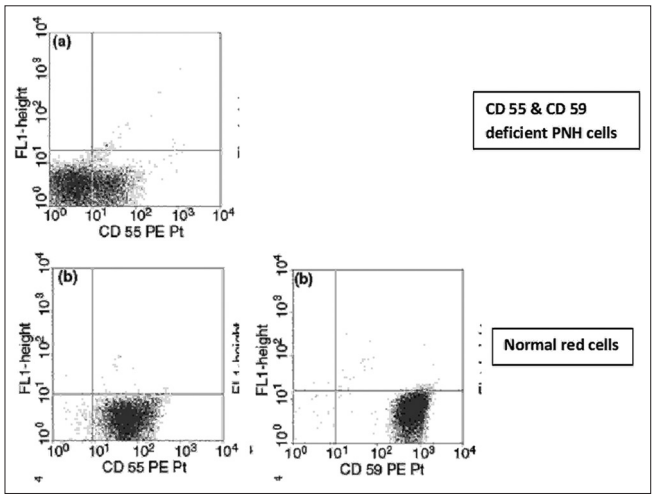

Figure 4: Flow cytometry in paroxysmal nocturnal hemoglobinuria

The surface of normal RhD-positive red cells expresses about 10,000–30,000 D antigens per cell while weak D red cells have antigen densities between 70 and 4000 D antigens per cell.[34] Red cells with <30 D antigen sites have now been shown to induce alloimmunization in RhD-negative individuals.[35-37] Several studies on the quantification of D antigens have been reported in normal D positive, weak D and partial D cases, and various Rh phenotypes.[38-41].

Various quantitative methods have been used for the estimation of D antigenic sites on red cells including the sensitive FC. FC is a valuable tool to study D antigens on red cell surfaces, as it is a rapid, reliable, and efficient method and can detect very small amounts of D antigens.[41]

Using FC, van Bockstaele *et al*. demonstrated the differences in the D antigen density of various Rh phenotypes but showed that Weak D (D^u) was not differentiated from D negative (cde/cde).[42] In an unpublished thesis work by Verma *et al*. from SGPGIMS it was possible to distinguish between Weak D and D negative antigen densities. The mean channel fluorescence (MCF) of D negative was 5.08 in contrast to the MCF of Weak D which was 15.27 ($p< 0.001$) [Figure 5]. The D antigenic sites (MCF) in weak D in their study were much higher compared to other studies.[43] However, the result of Verma *et al*. was found consistent with those obtained by Cunningham *et al*. who analyzed

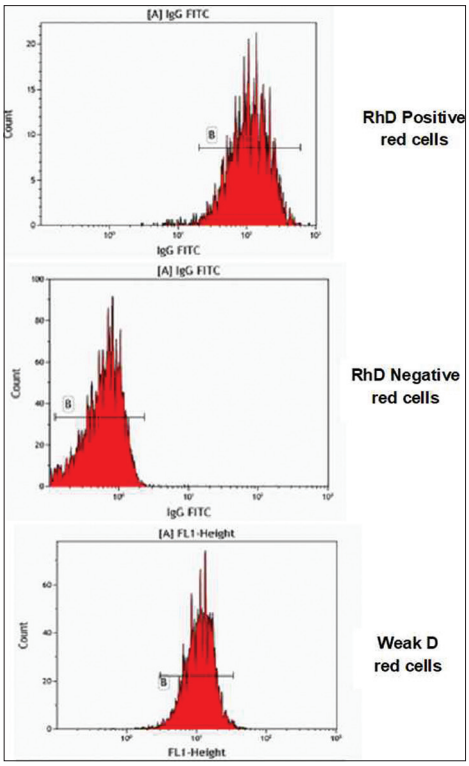

Figure 5: Flow cytometry in D antigen detection

the binding of polyclonal radiolabelled anti-D antibodies and showed that the number of antigenic sites differed by a factor of 10–15 between the D positive and the weak D (Du) red cells.[44] In the study by Verma *et al*. the MCF of D positive red cells was 108.06 in contrast to 15.27 in weak D. They could not actually calculate the density of D antigens as it required standard red cells with a known number of D antigens. They have calculated the MCF as an indirect indicator of D antigen density. This approach has also been used in previous studies.[43,45] Most authors concluded that evaluation by FC is less time-consuming, does not need radiolabeling, and could also be applied for routine analyses.[42-45]

Flow cytometry in platelet antibody detection and platelet immunology

More than 80% of platelet refractoriness (PR) are due to nonimmune causes such as splenomegaly, fever, sepsis, antibiotics, and disseminated intravascular coagulation.[46] Immune causes, occurring in <20% of the cases, involve alloimmunization against human leukocyte antigens (HLA) and, to a lesser extent, human platelet antigens (HPA) following exposure through transfusion, pregnancy, or transplantation. Among the immune causes, HLA antibodies are responsible for approximately 80%–90% of PR and HPA antibodies for approximately 10%–20% of cases.[47]

The immune causes of PR can be diagnosed by laboratory tests and testing for HPA antibodies is technically

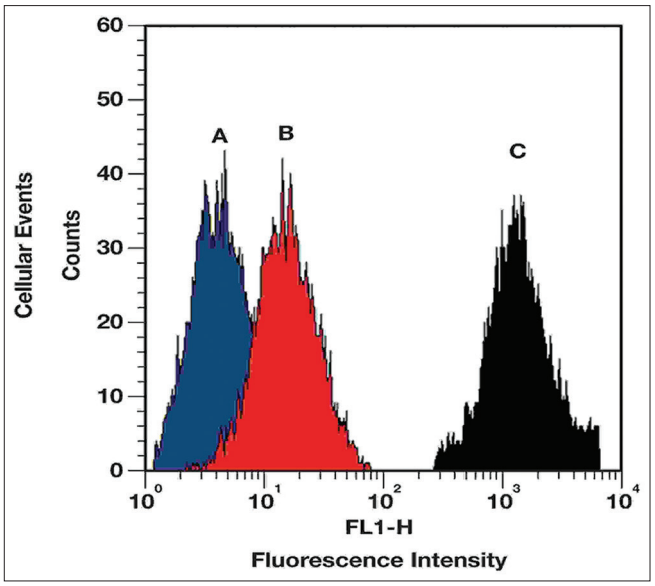

Figure 6: Flow cytometry in the detection of platelet antibodies

demanding. The platelet antibody detection tests available include microcytotoxicity and the platelet immunofluorescence test (PIFT) either by microscopy or FC. PIFT is the gold standard technique that permits the identification and quantification of platelet-specific antibodies however this technique is very laborious and time-consuming.[48-50] This typical assay involves incubating patient's serum with intact blood Group O platelets, followed by washing away unbound antibody and detection of platelet-bound antibodies with fluorescent-labeled anti-human IgG or immunoglobulin M (IgM) reagents by FC [Figure 6].[51]

Bub *et al*. found the FC-based PIFT to be efficient, fast, and feasible as an initial screening to detect platelet antibodies and a useful tool to crossmatch platelets for the transfusional support of patients with refractoriness. They investigated that the sensitivity of FC-PIFT was 86.11% and the specificity was 75.00% with a positive predictive value of 75.61% and a negative predictive value of 85.71%. The accuracy of the method was 80.26%.[52] FC has also been used successfully to detect autoantibodies in a direct assay of the patient's platelets.[53] Platelet autoantibodies testing in immune thrombocytopenia (ITP) can discriminate acute from chronic forms of the disease and is helpful in follow-up of patients. Determination of platelet-associated Ig (PAIg) like PAIgM in the combination of PAIgG has been found to be of interest in the investigation of ITP. Aref *et al*. concluded that FC is a sensitive method of the detection of platelet autoantibodies that could be used in the screening of suspected ITP [Figure 7].[54]

Regarding HLA antibody screening, Buakaewand Promwong observed that the FC technique detected antibodies more frequently than the lymphocytotoxicity test (LCT) and solid-phase red cell adherence

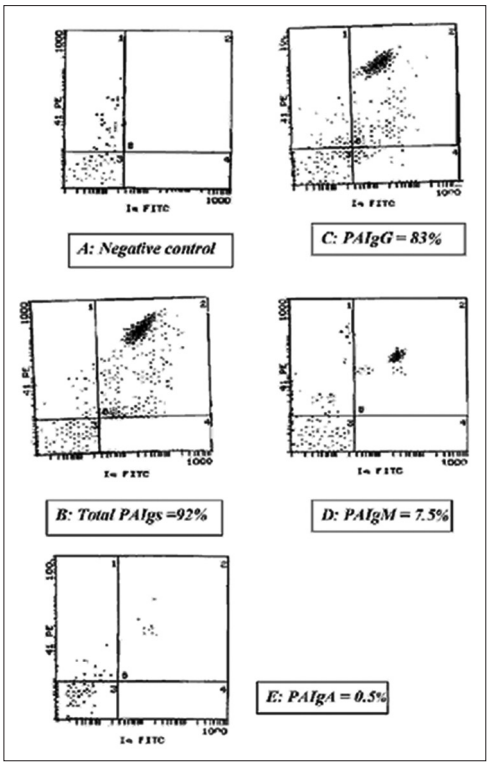

Figure 7: Flow cytometry in acute ITP = Immune thrombocytopenia

assay (SPRCA). In addition, the FC technique was found to be quick, required only a small amount of sample and was easy to perform and simple to interpret. They found the FC technique to be suitable for routine investigations of alloimmune causes of platelet transfusion refractoriness including neonatal alloimmune thrombocytopenia, post-transfusion purpura, and HLA antibody screening in a potential organ transplant recipient.[55]

Freedman and Hornstein compared LCT and SPRCA with the FC technique in platelet crossmatching and found that the FC technique had the best sensitivity and specificity.[56] Köhler *et al*. reported that sensitivity/specificity of FC technique was 94.7% and 96.3%, respectively, when monoclonal antibody-specific immobilization of platelet antigens (MAIPA) was taken as a reference indicating that sensitivity/specificity of FC is approaching that by the MAIPA assay.[57] This is a reason why most studies used FC as the reference method in the investigation of platelet antibodies.[57]

In addition, heparin-induced thrombocytopenia (HIT) can be diagnosed by flow cytometric detection of antiheparin/PF4 antibodies. Several publications have evaluated this method for the diagnosis of HIT.[58-60]

Conclusion

We conclude that today FC is used in both routine and research laboratories. In addition to the important application of FC discussed above many diverse roles of FC has been discussed in the literature [Table 1]. FC plays an important role in various investigations of patient samples and is a vital addition in research-focused studies. Flow cytometers are becoming smaller, cheaper, and more user-friendly and are available in many routine laboratories. Hence, the method should be considered a valuable tool to use in addition to serology and genomic typing. In addition, FC represents a highly innovative technique for many common diagnostic and scientific fields in TM. Finally, it is the tool of choice to develop and optimize new cellular and immunotherapeutic trials.

Table 1: Application of flow cytometry in transfusion medicine

Applications	References
DAT negative AIHA	[10,13,14]
Fetomaternal hemorrhage	[15-22]
Paroxysmal nocturnal hemoglobinuria	[23-27]
Quantification of RhD antigen	[32-45]
Platelet antibody detection and platelet immunology	[46-60]
Detection of Kell antigens	[61,62]
ABO expression and chimerism study	[63,64]
Platelet storage lesion	[65,66]
Evaluate residual leukocytes in blood components	[67,68]
Granulocyte/monocyte integrity and immunology	[69-72]
Hematopoietic stem cell transplantation and stem cell biology	[73-77]
Study of immune cells and immunotherapeutic trials	[78-81]

AIHA=Autoimmune hemolytic anemia, ABO=ABO blood group antigen, DAT=Direct antiglobulin test

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Conflicts of interest
There are no conflicts of interest.

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