REVIEW ARTICLE

Flow Cytometric Immunophenotyping of Acute Leukemia the Essential Considerations

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Abstract

Objective: This article serves to promote the interest of participants involved in management of hematological malignancies in the efficacy of flow cytometry in the immunophenotypic characterization of leukemias and lymphomas. This well-defined characterization plays a crucial role in diagnosis, classification, prognostic evaluation, and detection of minimal residual disease, in the context of clinical features and morphological diagnosis. **Methodology:** Relevant literature was retrieved to highlight the principles of operation of flow cytometry, applications, derivable clinical information, sources of errors, necessary antibodies and general considerations towards definitive diagnosis of acute leukemia. **Conclusion:** Hematological malignancy is an extremely heterogeneous disorder. Use of flow cytometric immunophenotyping in these disorders is already proven over two decades and allowed clinicians for proper classification along the latest WHO classification guidelines and will pave the way for targeted therapy and better patient management. The flow cytometric immunophenotyping services in the country will significantly outweigh the cost by reducing the duration of hospital stay and patient management in right way.

Key words

Flow cytometry, immuno-phenotyping, hematological malignancy, leukemia

Introduction

Hematologic malignancies are a group of neoplasms that arise through malignant transformation of bone marrow derived cells. There is great diversity in this group of disorders reflecting the complexity of normal hemopoiesis. These malignancies are clonal disorders of hemopoietic stem cells whereby an abnormal clone of cells proliferates autonomously, suppressing normal hemopoiesis, and also infiltrates tissues and organs, thereby disturbing their normal physiological activities. The fundamental defect is believed to be due to a genetic aberration at the level of the hemopoietic stem cell.

Hematologic malignancies, traditionally regarded as leukemias, lymphomas and myeloma, are in fact exceptionally heterogeneous, with WHO classification (2008) system recognizing over 60 different clinical and pathological disease subtypes.[1] This complexity is further reflected in widely varying clinical features, treatment pathways and outcome associated with these diseases. While some hematological malignancy is acute and can be rapidly fatal, if untreated, many others are insidiously progressive and become chronic, leading to eventual death. The acute types are often malignancies emanating from precursor cells while the insidious ones are often malignancies arising from relatively or fully matured cells.

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Flow cytometry (FCM) is a very robust and effective method for the diagnosis of hematologic malignancies and hence FCM immunophenotyping of hematologic malignancies is the most relevant clinical application of flow cytometry. Immunophenotyping of abnormal hematological cells is very useful for the diagnosis, classification, prognostic evaluation and detection of residual disease in patients with hematologic malignancies.[2-4] Applying the technique multiple characteristics on single cells can be analyzed and has become indispensable in view of its role in clearly defining the complex hemopoietic malignancies according to lineage and maturation stage, using differentiation markers. The procedure evolved over the last two decades from microscopic evaluation of antibody staining to flow cytometry, which is now recognized as the preferred method for data analysis.[5-8]

This review discusses the usefulness of FCM, current status of use of FCM in Bangladesh, general consideration to use it in the routine diagnostics and the panels of antibodies necessary to classify common hematological malignancies and finally hematological cases we diagnosed in Apollo Hospitals Dhaka in 2015-2016.

Historical background and current status of use of flow cytometry in Bangladesh

The application of flow cytometry in diagnostic hematopathology has gained much momentum since commercial availability of monoclonal antibodies and fluuorochromes in the late 1980s. FCM immunophenotyping is now an established and necessary laboratory test in the clinical evaluation of any suspected hematologic malignancy. Moreover, due to technological advances in both the hardware and software of FCM instruments three- and four-color FCM studies is being replaced by the six- to ten color studies in the routine laboratories.

In Bangladesh flow cytometry was first started in research for immunophenotyping of animal samples in ICCDR,B in March 2002 with FACS Calibur and continued till today with more advanced version with sorting function. Clinical use of flow cytometry for immunophenotyping of paediatric leukemias was first started with FC500, Beckman Coulter in BSMMU in 2007 which discontinued in 2011 due to shortage of technical hands. In 2012 Armed forces institute of pathology (AFIP) has started limited use of flow cytometry for immunophenotyping of leukemias with FC500 which is still in operation. Department of Microbiology, BSMMU started use of flow cytometry for immunophenotyping of leukemias with a research project in 2014 and then in 2015 started routine use of immunophenotyping of acute leukemias but discontinued within a year due to technical problems. Apollo Hospitals Dhaka has started flow cytometry for routine use of immunophenotyping of leukemias, lymphomas, plasma cell dyscrasias, immunodeficiency, PNH and stem cell count from 2015. Apollo Hospitals Dhaka is the first center in the country which has diversified the use of flow cytometry and arranged CME program on clinical application of flow cytometry in July 2015 where all the prospective leaders in the country such as BSMMU, DMCH, SSMCH, Dhaka Shishu Hospital, CMH, AFIP, ICDDRB joined.

Although advances in science and technology have created techniques for the molecular diagnosis of most of these hematologic malignancies in the developed world, in Bangladesh the diagnosis of hematologic malignancies has remained at a rudimentary level because hematologist in the country failed to carefully define their priorities and more inclined for outsourcing from Indian laboratories instead of giving effort for the acquisition of advanced technologies by our country people. Therefore, clinical features, morphology and occasionally limited immunohistochemistry remain our mainstay in diagnosing hematologic malignancies.

Application of Flow Cytometry

The usefulness of FCM immunophenotyping is multifold[9], as it facilitates (1) the distinction between neoplastic and benign conditions, (2) the diagnosis and characterization of lymphomas and leukemias, (3) the assessment of other neoplastic and preneoplastic disorders such as plasma cell dyscrasias and myelodysplastic syndromes, and (4) the detection of minimal residual disease in patients with acute leukemia or chronic lymphoid malignancies. In some groups of lymphoid neoplasms, FCM study also provides prognostic information.

FCM is useful for the enumeration of immune cells in primary immunodeficiency or secondary immunodeficiency such as in HIV infection. It has also become the preferred method to assess the number of hemopoietic progenitor cells (CD34+ cells) in cell products that will be used for bone marrow or peripheral blood stem cell transplantation.[10] Immunophenotypic enumeration of leukemic cells has both diagnostic and prognostic value. Flow cytometry is also being used in some routine labs for assessment of expression of glycosylphosphatidyl-inositol (GPI) anchors by CD55 and/or CD59 staining on RBC and WBC to establish diagnosis of Paroxysmal nocturnal haemoglobinuria (PNH).[11] Further, red cell membrane defects in hereditary spherocytosis (HS) can be easily diagnosed by FCM with dye eosin-5'-maleimide (EMA)[12], which binds specifically to the anion transport protein (band-3) at lysine-430. Immunoplatelet counting by flow cytometry is the current reference method recommended by The International Council for Standardization in Haematology (ICSH) and the International Society of Laboratory Hematology (ISLH) for the enumeration of platelets.[13]

The principles of FCM

FCM uses the principles of light scattering, light excitation and emission of fluorochrome molecules to generate specific multiparameter data from particles and cells in the size range of 0.5 μm to 40 μm diameter.[14] The cells are hydrodynamically focused in a sheath of phosphate buffer saline before intercepting an optimally focused light source. Lasers are most often used as light source in FC. As the cells of interest intercept light, they scatter it, and fluorochromes are excited to a high energy state. This energy is released as photons of light with specific spectral properties unique to different fluorochromes.

One unique feature of FCM is that it measures fluorescence per cell or particle. This contrasts with spectrophotometry in which the percent absorption and transmission of specific wave lengths of light is measured for a bulk volume of sample. The scattered and emitted light from cells and particles are converted to electrical pulses by optical detectors.

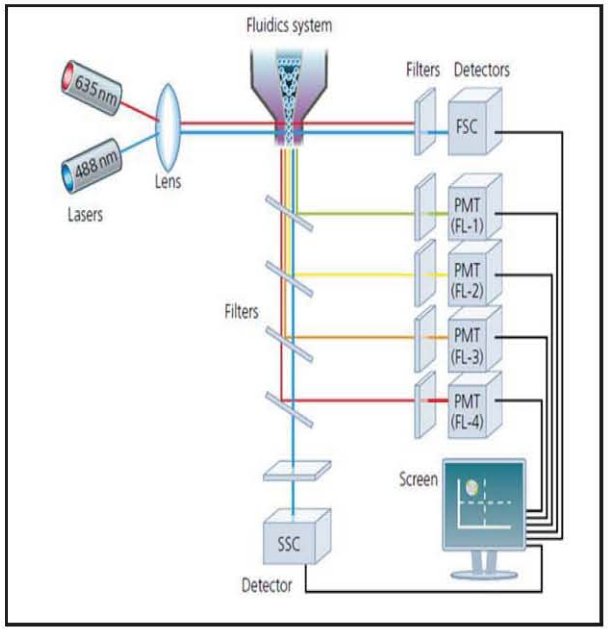

Fig 1. Schematic overview of typical flow cytometer setup

Collimated light is picked up by confocal lenses focused at the interception point of the cells and the light source. Light is sent to different detectors by using optical filters. For example a 525 nm band-pass filter placed in the light path prior to the detector will allow only green light to pass into the detector.

The most common type of detector used in flow cytometry is the photomultiplier tube (PMT). The electrical pulses originating from light detected by the PMT are then processed by a series of linear and log amplifiers. Logarithmic amplification is most often used to measure fluorescence in cells. This type of amplification expands the scales for weak signals and compresses the scale for strong or specific fluorescence signals.

After the different signals or pulses are amplified they are processed by an analog to digital converter which in turn allows for events to be plotted on a graphical scale. FCM data outputs are stored in the form of computer files using FCS 2.0 or 3.0 standards. Data corresponding to one sample can be stored as a list mode file and/or histogram file.

Approach to flow cytometry: Essestial considerations

A comprehensive approach to FCM data analysis and interpretation is necessary for proper diagnosis.[15] In the authors' experience, the optimal method is for the laboratory medical staff to apply a visual approach to FCM data analysis rather than relying on percentages. In other words, data interpretation is based on a visual appraisal of the FCM graphics, assessing the complex patterns formed by the shape and relative position of the cell clusters observed on various dot plots such as FSC versus fluorescence, side scatter (SSC) versus CD45, and correlated fluorescence dot plots. Any other analysis rather than relying on percentages. In other words, data interpretation is based on a visual appraisal of the FCM graphics, assessing the complex patterns formed by the shape and relative position of the cell clusters observed on various dot plots such as FSC versus fluorescence, side scatter (SSC) versus CD45, and correlated fluorescence dot plots. Any other approach to FCM data interpretation, using a scoring system or percent positive per antibody, underutilizes the full potential of FCM. Laboratory professionals, as well as clinicians, should realize that visual FCM data analysis is a process reminiscent of the microscopic examination of morphologic material (e.g., bone marrow aspirate smears, lymph node sections) in which the data form a pattern and are reported in a qualitative and quantitative (where appropriate) format.

Although microscopic examination encompasses all elements in the sample, reporting the data focuses only on the abnormal component. Similarly, the FCM interpretative report should be based on the cells of interest, even though the list mode data should be collected unselected (i.e., it includes all cells in the sample).

Interpretation of the FCM immunophenotyping results is one step in the diagnosis of malignant lymphoma and leukemia. Although, in many cases the diagnosis is apparent after a visual inspection of the FCM immunophenotyping data together with the DNA cell cycle histogram, in other instances the antigenic profile and the pattern of the cell clusters suggest only a differential diagnosis instead of a specific disorder. In such cases, it is critical that the diagnostic interpretation takes into account the other clinical and laboratory data, such as the hemogram findings and the cytologic/morphologic features. The synthesis of the pertinent results requires the responsible medical staff in the laboratory to be well versed in the different subdisciplines of hematopathology. Irrespective of whether a case is straightforward or complex, the authors advocate a routine systematic approach to FCM diagnostic interpretation. This will ensure that no relevant information is omitted.

A correlation between the FCM findings and the available morphologic data should be performed in all cases. Wright-Giemsa-stained cytospins made from the cell suspension of the tissue or fluid submitted for FCM study must be reviewed, to correlate the findings with those derived from the FCM plots. This is especially helpful when abnormal (neoplastic) cells are few or the FCM data cannot be clearly interpreted.

For peripheral blood specimens, the FCM data are correlated with the hemogram and cytologic features from a fresh blood film. Similarly, FCM interpretation on bone marrow specimens should include a review of the hemogram, peripheral blood film, bone marrow aspirate smear or imprint, and cytochemistries, where appropriate. It cannot be emphasized enough that hemogram findings, along with fresh peripheral blood and bone marrow smears, must accompany the specimen when bone marrow is sent to a referral laboratory for immunophenotyping, so that a proper, thorough diagnostic evaluation of the case can be conducted. In addition to the above-mentioned minimum correlation with the morphologic findings, it is also important to review immunoelectrophoresis results in suspected lymphoplasmacytoid neoplasms or plasma cell dyscrasias.

Pre-analytical factors for blood and bone marrow samples are that: (a) anticoagulant (EDTA or heparin) be used; (b) the specimen is analyzed within 24 hours; (c) storage should be at 2°C–20°C; (d) there is no need to isolate mononuclear cells; and (e) great care be taken with lysing procedures. It is recommended that immunophenotyping should be assessed in conjunction with clinical features and cell morphology with strict adherence to quality system essentials, and careful selection of fluorochromes: antibodies whose normal expression is rather dim (eg, CD7, CD10, CD11b, CD13, CD2, CD34, CD64, CD117 or Tdt) should be labeled with the brightest fluorochromes like phycoerythrin and, if instrumentation allows, allophycocyanin.

Extensive studies[2-7] have been done on the factors that affect the results of immunophenotyping, including:

1. The type and quality of samples.
2. The reagent and sample preparation protocols.
3. Instrument set-up and calibration.
4. Potential components of subjectivity introduced during data analysis or with the interpretation of the results represent the most common sources of variability.

Lineage assignment of pathological cells

When FCM is needed for lineage assignment is depicted in table I. Markers used for specific lineage is mentioned below:

1. Acute lymphoblastic leukemia, B-lineage is defined by the expression of at least two of CD19, CD22, CD79a, CD10 in the absence of specific T-cell and myeloid markers.
2. Acute lymphoblastic leukemia, T-lineage is defined by the expression of cytoplastic and/or surface CD3 in the absence of specific B-cell and myeloid markers.
3. Acute myeloid leukemia is defined by the expression of at least two of MPO, CD13, CD33, CD117, in the absence of specific T-cell and B-cell markers. Further, myeloid differentiation is defined by CD15, CD65 and monocytic differentiation by CD14, CD64, CD4, CD11c, Lysozyme. Erythoid precursor is defined by CD235a, CD71 and megakaryocyte precursor is by at least one of CD41, CD42, CD61.

Table I Role of FCM in Lineage assignment

- Morphologically lymphoid, MPO negatice
  - FCM to confirm lymphoid lineage and rule/out AML MO
- Morphologically myeloid/unequivocal, MPO negative
  - FCM essential for lineage assignment
- Morphologically myeloid, Auer Rods positive
  - FCM recommended for aberrant marker expression before starting therapy
  - Morphologically myeloid, with <10% MPO positivity
  - FCM recommended to rule out B-ALL before starting therapy
- Morphologically suspicious of megakaryoblastic leukemia
  - FCM essential to confirm megakaryocytic lineage
- Acute leukemia-relapse (esp. on therapy)
  - FCM essential to establish lineage of blasts

Normal peripheral blood and normal bone marrow

A normal-appearing specimen (Fig. 2) by FCM analysis is defined by the absence of abnormalities. The following should not be present in normal specimens: (1) an increased number of blasts; (2) a marked preponderance of a specific cell population (lymphocytes, monocytes, granulocytes, red cell precursors, or eosinophils); (3) abnormal B-cells, T-cells, or plasma cells; (4) abnormal "antigenic" maturation in the granulocytic elements; and (5) nonhematopoietic elements. The size of each cell cluster only reflects its relative proportion. Therefore, the evaluation needs to take into account the CBC and bone marrow cellularity, if the specimen is marrow aspirate. For peripheral blood specimens, the cell populations are assessed in light of the total WBC count. The bone marrow FCM picture in some neoplastic disorders such as low-grade MDS (e.g., refractory anemia) or myeloproliferative disorders (MPDs) (e.g., polycythemia rubra vera [PRV] or essential thrombocythemia [ET]) can be indistinguishable from that in a normal subject.[21]

In normal specimens, the blast region is essentially empty, with only a few scattered events present. Enumeration of the blast events can be achieved by drawing a gate around this region (Fig.3, blue circle). In the blood, the presence of a discrete cluster of blasts, however tiny it may be, is a significant finding. In the bone marrow, the threshold generally employed is that which has been traditionally accepted for clinical remission of acute leukemias (i.e., less than 5% myeloblasts).

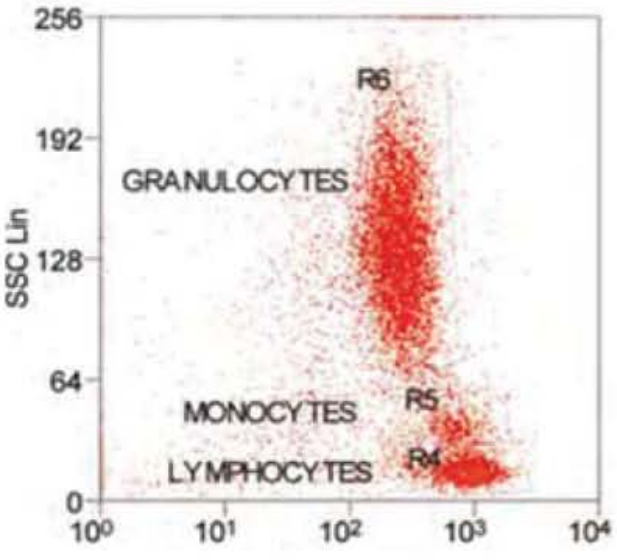

Fig. 2: Cell clusters in normal peripheal blood and bone marrow on SSC/CD45 gating

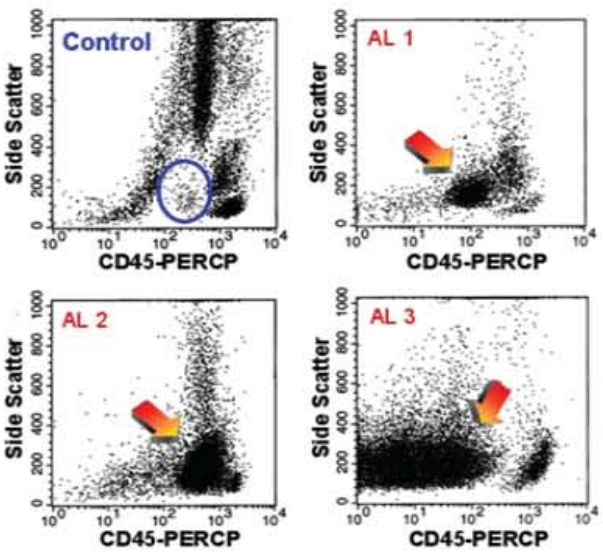

Fig. 3 Typical dot blot display in healthy control and acute leukemia (AL)

However, because of the manner in which the bone marrow is collected in most institutions, the aspirate sample allocated for FCM analysis is often much more hemodilute than the sample spread on the smears. Therefore, a bone marrow blast level of less than 5% in the FCM sample does not necessarily exclude a neoplastic condition. The presence of a discrete blast cluster in the bone marrow, even without phenotypic aberrancies and at a level considered within the normal range, can still be cause for concern. Knowledge of the antecedent clinical history and close follow-up are warranted in such cases to exclude or confirm either preneoplastic conditions (MDS) or minimal residual AML.[22]

Diagnosis of acute leukemia and role of FCM

Diagnosis of acute leukemia is typically made on clinical features and morphological examination of peripheral blood and/or bone marrow where blasts should be more than 20% with the exception of AML with recurrent cytogenetic abnormalities where blasts can be below 20%.

Bone marrow specimen is always diluted by blood percentage of blast cell count by FCM is relative and may under represented. Blast count on peripheral is more realistic than bone marrow however; it can also be under represented by the non-lysed RBC and cellular debris events. FCM is used to confirm the presence of blasts by immaturity markers CD34, TdT and CD117 and dim expression of CD45. Further, FCM will determine lineage (B, T-lymphoid or myeloid), determine immunological subtype, presence aberrant phenotypes, and customize follow up panel for minimal residual disease.

Antibody panel design and cases sub-typed in Apollo Hospitals Dhaka

For more than two decades, immunophenotyping has been providing relevant information for the diagnosis, classification and monitoring of hematological malignancies. For the analysis of leukemias and lymphomas, the antibodies are assembled into panels. Most consensus leukemia & lymphoma antibody panels consist of lists of markers based on expert opinion. We are using following antibody panels to diagnose and classify acute leukemias: CD45, CD3, CD4, CD5, CD7, CD8, CD10, CD13, CD14, CD15, CD19, CD33, CD34, CD64, CD117, HLA-DR, MPO, CD79a, cCD3. When necessary, stain with erythrocyte marker CD235a (glycophorin-A) and megakaryocyte marker CD41 is done later. Further to reduce the cost of the test a basic panel is designed with CD45, CD5, CD7, CD10, CD13, CD19, CD33, CD34, CD117, HLA-DR, MPO, CD79a, cCD3. For CLL we are using CD45, CD3, CD5, CD10, CD11c, CD19, CD20, CD23, CD25, CD38, CD56, CD103, CD200, FMC-7, anti-kappa, anti-lambda. For multiple myeloma we are using CD45, CD38, CD138, CD19, CD56, anti-kappa, anti-lambda. Using these panels we have already categorized 166 cases of acute leukemia suspected patients and 20 chronic lymphocytic leukemia (CLL) cases and 13 multiple myeloma (MM) cases (Fig. 4).

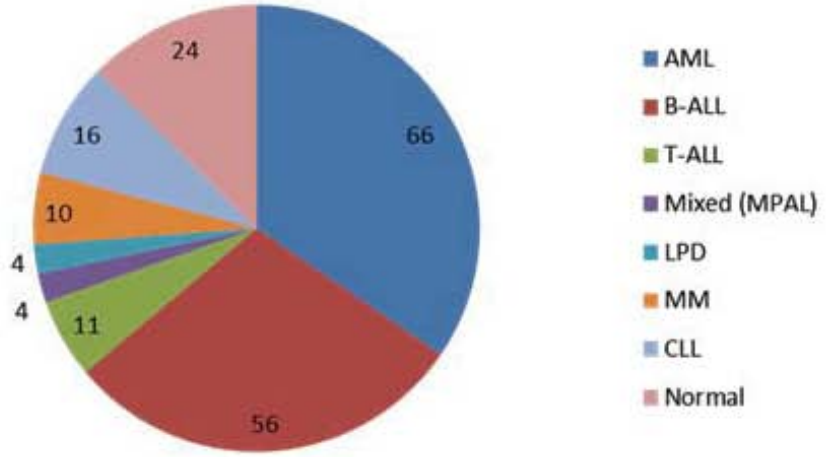

Fig. 4: Sub-types of Cases Diagnosed by FCM in AHD

Conclusion

To meet the standard care in the management of hematologic malignancies, a giant stride will have to be taken not only to improve manpower development in cancer diagnosis and treatment but also in equipping our specialist hospitals with modern equipment of which flow cytometry is an essential part. Smaller, less expensive instruments and an increasing number of clinically useful antibodies are creating more opportunities for routine clinical laboratories to use flow cytometry in the diagnosis and management of disease. It is very important to apply essential considerations for proper typing of malignancies by flow cytometry. At the same time, all requirements for efficient and continuous functioning of equipment needs to be ensured.

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