Review Article

# Advances and issues in flow cytometric detection of immunophenotypic changes and genomic rearrangements in acute pediatric leukemia

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**Abstract:** Flow cytometry with its rapidly increasing applications has been using to aid the diagnosis of hematological disorders for more than two decades. It is also the most commonly used technology in childhood leukaemia diagnosis, characterization, prognosis prediction and even in the decision making of targeted therapy. Leukemia cells can be recognized by virtue of unique cell marker combinations, visualized with monoclonal antibodies conjugated and detected by flow cytometry. Currently, such instruments allow the detection of eight or more markers by providing a comprehensive description of the leukemic cell phenotype to facilitate their identification, especially in detecting and monitoring of minimal residual disease (MRD) during treatment. Additionally, the flow cytometric DNA index (DI) can identify biclonality at diagnosis and distinguish persistent aneuploid leukemia during induction therapy, when the standard cytogenetic and morphologic techniques fail to do so. This review focuses on the latest advances and application issues about some of flow cytometric diagnostic and prognostic applications for acute pediatric leukemia.

**Keywords:** Flow cytometry; acute pediatric leukemia; immunophenotyping; minimal residual disease (MRD); DNA index (DI)

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## Introduction

Pediatric leukemia is the most common type of cancer in children. Around 95% cases of pediatric leukemia are acute. Acute leukemia comprises a heterogeneous group of malignant diseases characterized by clonal expansion of immature hematopoietic precursor cells. Two major categories of acute leukemia are recognized: (I) acute lymphoblastic leukemia (ALL) subdivided into B- and T-cell precursor ALL and (II) acute myeloid leukemia (AML) characterized by an overproduction of immature myeloblasts or leukemic blasts (1). ALL covers approximately three out of every four cases of childhood leukemia while AML is the next most common type.

In pediatric patients with acute leukemia, diagnosis and treatment decisions are based on the status of peripheral blood and bone marrow cellularity. Historically, identification of leukemic cells among normal bone marrow cells has relied on their morphology. However, the reliability of morphologic examination of peripheral blood and bone marrow largely depends on the hematologist's expertise, and its sensitivity is fundamentally limited by the similarities in appearance between leukemic cells and normal lympho-hematopoietic progenitors affecting the effective treatment plan making.

Due to the limitation of morphologic assessment, immunophenotyping diagnostic cells and their potential normal counterparts using flow cytometry originated in the late 1980s. Immunophenotyping is a technology used to study antigens expressed on cell surfaces to determine cell type and stage of differentiation. This technique is commonly used in basic research. Diagnosis of leukemia involves the labeling of white blood cells from blood, bone marrow or spinal fluid with antibodies directed against surface proteins. By choosing appropriate antibodies, the definition of leukemic cells can be accurately determined. The labeled cells are processed in a flow cytometer, a laser-

based instrument capable of analysing thousands of cells per second. The whole procedure can be performed in a matter of a couple of hours.

Immunophenotypic similarities between the tested cells and their potential normal counterparts allow the assignment of such cells to a given hematopoietic cell lineage and maturational stage, as well as the identification of aberrant phenotypes, such as leukemia-associated immunophenotypes, which can be reliably recognized by flow cytometry. In fact, for more than two decades, immunophenotyping has been providing key information for the diagnosis, classification and monitoring of leukemia and allowing their detection of very small numbers, whose recognition may be impossible by morphologic examination (1-9).

Measuring response to chemotherapy is the backbone of the clinical management of pediatric patients with acute leukemia. The concept that patients with leukemia in morphologic remission could have measurable levels of minimal residual disease (MRD) was first demonstrated in the early 1980s (10). Similar to immunophenotyping, flow cytometric MRD analysis relies on the detection of surface phenotypes unique to leukemia cells, but not present on normal hematopoietic cells. The sensitivity can be routinely achieved to the detection of 0.01% (11). The levels of MRD are now widely used as parameters for therapy efficacy and risk assignment in ALL, and increasingly so in AML (11-13).

The detection of chromosomal abnormalities has important diagnostic and prognostic significance in acute leukemia. Apart from karyotyping of cytogenetic analysis, flow cytometric measurement of DNA index (DI) has been shown to play an important role in the characterization of the leukemic clones and has been used as a prognostic factor in childhood ALL (14,15).

## Immunophenotyping using flow cytometry in acute childhood leukemia

Leukemia cells can be recognized by virtue of unique cell markers visualized with monoclonal antibodies and flow cytometry. Together with cytomorphology and cytochemistry, immunophenotyping by flow cytometry is crucial for the detection and lineage assignment of blast cells in diagnostic samples, including the definition of acute leukemia of ambiguous lineage (16,17). Comparison of the immunophenotypic features of blasts cells versus normal hematopoietic precursors and immature cells contributes to the definition of the stage of maturation arrest of the blast population within the B- and T-lymphoid lineages as well as the neutrophilic, monocytic, megakaryocytic or erythroid lineages.

Such immunophenotyping requires careful selection of unique combinations of individual markers, based on their degree of specificity for the identification of a given cell lineage, maturation stage and aberrant phenotype, as well as the selection of appropriate antibody clones and fluorochrome conjugates to be used in multicolor combinations. The performance of these marker combinations is even more relevant than that of the individual markers. Consequently, such careful selection of reagents is essential for the design of standardized multicolor antibody combinations that provide unique staining patterns for each normal, or aberrant, cell population in a given sample (18-20).

Although immunophenotying by flow cytometry has become standard practice in the evaluation and monitoring of patients with acute leukemia, considerable variability continues to exist in reagents used for evaluation and the format in which results are reported. Several committees have attempted to define consensus sets of reagents suitable for general use in the diagnosis and monitoring of acute leukemia. In 2007, the Bethesda International Consensus first successfully defined a set of consensus reagents suitable for the initial and secondary evaluation of each cell lineage of leukemia cells (20). In 2012, the EuroFlow group published a set of 8-color antibody panels for the diagnosis and classification of acute leukemias. The panels are designed in a flexible way to fit the needs of distinct diagnostic laboratories and they can be applied in one or multiple sequential steps. Depending on the precise clinical question associated with a sample suspected of containing blast cells, the first step includes a single 8-color tube, the acute leukemia orientation tube (ALOT), complemented by a multi-tube panel designed for full characterization of the malignancy. The choice of the second panel depends on the results obtained with the ALOT, that is, the antibody panel for confirmation and classification of B-ALL, T-ALL, or the antibody panel for non-lymphoid acute leukemia, the so-called AML/myelodysplastic syndrome panel. Rare cases of ambiguous lineage leukemias identified with the ALOT then require the use of more than one complementary panel (19). This flexible 8-color antibody panel for multidimensional identification and characterization of normal and aberrant cells are optimally suited for immunophenotypic screening and classification of hematological malignancies (19). Together with standardization of flow cytometer instrument settings and immunophenotying protocols (18), the EuroFlow antibody panels can be considered as the standard approach for standardized multidimensional flow

cytometric immunophenotyping for diagnostic screening and classification of hematological malignancies (19).

## MRD identification using flow cytometry in acute pediatric leukemia

The prognostic significance of MRD in pediatric ALL was demonstrated in many studies involving newly diagnosed patients, patients with first-relapse ALL, and those undergoing hematopoietic stem cell transplant (21-31). Evidence has also accumulated in AML, with several studies reported of the significant association between MRD and relapse (32-34).

The introduction of methods for MRD detection has revolutionized monitoring of treatment response in acute leukemia. These methods can not only recognize leukemic cells by objective criteria, thus potentially improving the reliability of blood and marrow examination, but they also allow the detection of leukemic cells well beyond the resolution of microscopic examination.

Traditional morphologic assessment has limitation in sensitivity in MRD detection. Bone marrow samples collected after a temporary stop in chemotherapy, after the end of treatment, or after hematopoietic stem cell transplantation, may contain a high proportion of recovering immature lymphoid cells whose morphology resembles that of ALL lymphoblasts (35). Therefore, morphologic assessment of these samples is difficult and may result in erroneous conclusions. The application of flow cytometric MRD assays can clarify the identity of the morphologically ambiguous cells, where these cannot be detected by morphology or other techniques. In a study performed with 248 bone marrow samples collected after two weeks of remission induction therapy from children with newly diagnosed ALL, result showed only 12.9% had leukemic lymphoblasts identifiable by morphologic analysis and all of these had at least 0.01% cells expressing leukemia-specific immunophenotypes (24). In two samples with 9% and 16% leukemic cells by flow cytometry, in contrast, the morphologic analysis revealed only apparently mature normal lymphocytes (24). Therefore, patients in complete morphologic remission may still have a large number of residual leukemic cells.

In the last decade, the detection of MRD by flow cytometric or molecular techniques has come to be recognized as one of the most important clinical measures and is now routinely evaluated in experimental clinical treatment protocols. A study of 129 samples with MRD ≥0.01% showed an excellent correlation between the results of the two methods (36). Kerst *et al.* analysed 105 samples from 30 patients with ALL and also found highly concordant results for these two methods (37). Irving *et al.* studied MRD from 134 patients enrolled in the UKALL 2003 trial on day 28 (end of remission induction) and week 11 (completion of consolidation). Overall, 115 samples including 90 MRD 0.01% and 25 MRD ≥0.01% were measured by both methods. Most of the 19 discordant samples were around the threshold level and MRD was detectable by both techniques (38). With the improvement in methodologies, the concordance between MRD assays should improve. In patients with ALL, flow cytometry and PCR amplification of antigen-receptor genes provide similar results, if MRD is present at the levels of 0.01% or above, and hence the choice between these two methods is primarily dictated by the facilities and expertise available. In comparison with molecular techniques, the flow cytometric detection of MRD has the advantage of general applicability, high speed and lower cost, and hence has been the preferred method used for MRD detection by many laboratories. However, the sensitive flow cytometric detection of MRD requires evaluation of a suitably large number of cells, roughly 1,000,000 cells in order to achieve a sensitivity of 0.01% of white cells. In the St Jude Total XV study, MRD could be monitored by flow cytometry with sensitivity of 0.01% in 482 of 492 patients (98%) (39).

The targets most frequently used to monitor MRD in AML are transcripts originating from gene fusions, mutations, or overexpression, and leukemia-associated immunophenotypes (13,40). Flow cytometry is the only method that can be applied to monitor MRD in the majority of patients with AML. Studies on MRD by PCR amplification of fusion transcripts can only be used in a fraction of children with AML and results are difficult to interpret (41). More importantly, MRD measured by flow cytometry was a significant predictor of relapse, regardless of the morphologic results (41), could be performed reliably and was strongly correlated with clinical outcome (22,39).

Interestingly, specific immunophenotypic profiles have been associated with prognosis and/or unique cytogenetic and molecular abnormalities (42-45). Expression profiling found the gene for CD44 to be one of the best, correlating with the MLL genotype and with the subgroup of T-ALL patients, who later developed hematological relapse (45). In addition, dual $CD27^{pos}CD44^{pos}$ blasts are typically seen in $BCR/ABL^{pos}$ ALL and a subset of $TEL/AML1^{pos}$ patients exists with $CD44^{pos}CD27^{pos}$ blasts (45). In 74 cases with B-ALL children, including 21 cases with chromosomal translocations, $t(12;21)^{pos}$ and 53 cases with chromosomal translocation, $t(12;21)^{neg}$. The $t(12;21)^{pos}$ ALLs displayed a higher intensity of CD10 and HLADR expression together

 

with lower levels of the CD20, CD45, CD135 and CD34 antigens as compared to the t(12;21)$^{neg}$ cases (42). This immunophenotypic approach used for the identification of t(12;21)$^{pos}$ cases can be achieved with a sensitivity of 86% and a specificity of 100% (42). Moreover, a study with 82 B-ALL cases showed that BCR/ABL$^{pos}$ B-ALL cases constantly displayed a homogeneous expression of CD10 and CD34, but low and relatively heterogeneous CD38 expression, together with an aberrant reactivity for CD13 (43).

Flow cytometric immunophenotyping has proven to be of great utility for sensitive detection of low levels of residual blast cells and their distinction from normal regenerating immature cells in the bone marrow of acute leukemia patients during treatment (46). Flow cytometry is capable of detecting a single leukemia cell among 10,000 or more normal cells in peripheral blood during treatment for newly diagnosed T-lineage ALL in children (47). However, it is critical that flow cytometric analysis of MRD relies on markers that truly distinguish ALL cells from normal cells, including lymphoid progenitors; otherwise, the risk of false-positive MRD results is high. Therefore, a combination of flow sorting of small immunophenotypically defined cell populations with subsequent analyses of leukemia associated cytogenetic and molecular markers may provide a more sophisticated method for detecting low MRD levels.

## DI measurement in childhood ALL

Among age two to ten years old acute leukemia patients, hyperdiploid leukemia is recognized as an independent indicator of good treatment outcome (14). There is a covariation between modal chromosome number and traditional clinical risk factors, that is, the group with >51 chromosomes is associated with favorable clinical features, but the hypodiploid group forming 1-3% of the cases has no distinct clinical features (48). Therefore, when stratifying patients into future treatment protocols it will be important to reliably decide ploidy of the leukemic cells at diagnosis.

There are two major conventional techniques to investigate the ploidy of leukemic blasts. The first traditional way is the karyotyping of cultured bone marrow cells with light microscopy counting of Giemsa-banded metaphase chromosomes (49). However, the low number of metaphases studied required, making this technique insensitive, it also depends on a successful cell culture. The second way is the DNA content measurement by image analysis or by flow cytometry (50,51). The DNA content of cells is measured by the ability of propidium iodide to bind stoichiometrically to DNA under appropriate staining conditions. The nuclei of these stained cells are evaluated individually for DNA content by flow cytometry. The results are displayed graphically as a histogram, in which the fluorescence emitted by each nucleus is directly proportional to its DNA content. The difference in DNA content can be expressed as the ratio of leukemia sample/standard DNA fluorescence, defined as the DI.

The highly significant correlation between modal chromosome number by karyotyping and DI by flow cytometry was shown in a study on 112 childhood ALL patients on fresh or frozen samples (15) and a study of 82 consecutive children with ALL (14). DI was also repeatedly found to be more sensitive than karyotyping in discovering small aneuploidy clones (14,15). In 7 of 19 childhood ALL cases, DI detected an aneuploid leukemic clone at day 15, and at day 29, whereas the karyotype in all these follow-up samples were diploid (14). Additionally, two patients were shown by karyotyping to have undetected biclonality at diagnosis and, in >20% of the aneuploid patients, the abnormal clone was revealed by DI during the first month of induction therapy, but showed a diploid karyotype (14). Furthermore, the cytogenetic approach only detected the hyperploid clone in three patients who presented the near-triploid/hyperploid entity, whereas the DI identified a minor population of hypodiploid cells besides the major hyperdiploid clone, thereby validating the diagnosis of severe hypodiploidy (15). However, the capacity to detect small DNA content abnormalities is dependent on the quality of the sample, the staining technique and the instrument used. In contrast, traditional karyotyping identifies smaller structural and numerical DNA changes.

DI is a prognostic factor in childhood ALL. A group of investigators have provided arguments that justify the use of DI measurement for evaluating the prognosis (52,53). A statistically significant difference in survival was found using the DI approach, while a difference was not found using modal numbers obtained by karyotyping. In childhood ALL, a DI of ≥1.16 is associated with hyperdiploidy of >50 chromosomes, approximately representing 25-30% of childhood ALL, has more favorable presenting features and higher cure rates than other major prognostic subgroups (15). On the other hand, a hypodiploid clone (<44 chromosomes) is associated with a poor prognosis (54).

The flow cytometric DI is technically fast on fresh or frozen samples. If the karyotype is essential to analyze chromosomal abnormalities, DI provides complementary information in aneuploid ALL, either by confirming the cytogenetic data, or by detecting additional clones not

 

identified when only using cytogenetic data.

## Current limitations

Flow cytometry is a rapid, cost effective, informative, sensitive, accurate qualification method, which is applicable to a wide range of disorders, especially in hematopoietic malignancy. The current limitations of flow cytometry include the requirement for consistent flow technique, lack of expertise in sample processing and subjective data interpretation and poor standardization across institutions. Advancing techniques will be further improved by standardizing the setting up of instruments, staining protocols and data analysis. These developments will make flow cytometry even more accessible within clinical applications.

## Future directions

Flow cytometry has developed rapidly since 1980s and become a mainstay of the modern clinical pathology laboratory, especially in leukemia diagnosis. Methods to study acute leukemia by flow cytometry are constantly being refined by the introduction of new markers, which take advantage of the capacity of newer instruments to detect an increasingly higher number of fluorochromes. New technologies, including mass cytometry (55) (spectrometry-based detection of elements conjugated to antibodies) and image cytometry (56) combining features of flow cytometry and imaging, can further increase this capability. This enhanced capability will facilitate the discrimination between normal and leukemic cells, increase the sensitivity of leukemia cell detection and will also allow the study of biologic features of leukemia cells, such as expression of molecules related to proliferation, apoptosis, signaling, and drug resistance. In addition, the traditional ways to analyze flow cytometric data will be inadequate when applied to the amount of information acquired with contemporary instruments and hence a parallel development in analytical software must take place.

Along with technology and instrumentation development, improved leukemia classification and tailoring of therapy will greatly improve patient outcome particularly for children with acute leukemia.

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## Footnote

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