

## DIAGNOSIS OF ACUTE LEUKEMIA BY FLOWCYTOMETRY IN POPULATION OF TRIPURA, NORTH-EAST INDIA

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### HOW TO CITE THIS ARTICLE:

Arup Bhaumik, Shikha Das, Sandeep Roy Sarkar, Payel Chakraborty, Biswajit Chowdhury. "Diagnosis of Acute Leukemia by Flowcytometry in Population of Tripura, North-East India". Journal of Evidence based Medicine and Healthcare; Volume 1, Issue 10, November 10, 2014; Page: 1320-1328.

**ABSTRACT:** The aim of this study was to diagnose acute leukemia in the population of Tripura using 3-lasers 7-color Flowcytometer. Immunophenotyping was done on peripheral blood samples of acute leukemia cases, by using 2-tube basic AML antibody panel. Samples collected from Regional Cancer Centre, Agartala included in this study 15 Acute Leukemia cases, reported during 2013 & 2014. Among them, 4 were ALL and 11 were AML cases. Out of 15 cases, 12 were adults (9 men and 3 women ranging from 17 to 77 years of age) and 3 were from pediatric patients. A strong correlation ( $r=0.92$ ) is obtained between the blast cell count by microscopy & flowcytometry. Moreover, this Immunophenotyping procedure on peripheral blood samples also allowed an efficient discrimination between the various cell lineages and facilitated the accurate leukemic blast cell percentage.

**KEYWORDS:** Acute Myeloid Leukemia, Acute Lymphoid Leukemia, Immunophenotyping, Flowcytometry.

**INTRODUCTION:** Flowcytometry is a powerful tool in the diagnosis and management of congenital and acquired immunodeficiency syndromes. It is also used for the diagnosis of leukemia and lymphomas. The characterization of acute leukemia is based on a multi parametric analysis which includes clinical features, cell morphology, and immunological markers. These markers have been shown to be important for the diagnosis and prognosis of acute lymphoid leukemia (ALL) and acute myeloid leukemia (AML) particularly when the precise lineage of the blast cells cannot be defined by microscopy morphology and cytochemistry.<sup>[1-4]</sup>

Immunophenotyping is a widely used method to diagnose and classify acute leukemia, thereby complementing morphology and cytochemistry.<sup>[5-7]</sup> Flowcytometric Immunophenotyping has a well-established role as a diagnostic modality in acute leukemia, particularly as a tool for assigning lineage and facilitating further pathologic classifications.<sup>[8-11]</sup>

In this study, at first we applied the Surface Staining by Lyse–Wash method and then analyzed the white blood cells of peripheral blood samples by Immunophenotyping. With the systematic use of leukocyte common antigen (CD45) marker in combination with lineage-specific markers, a good discrimination could be achieved between the blast cell populations and the normal cells. Here we also compared the data of light microscopic count and Flowcytometric count by statistical analysis.

**MATERIALS & METHODS:** The study cases of Acute Leukemia included 3 women, 9 men and 3 pediatric patients. Their age ranged from 3 to 77 years (Median= 33). 15 peripheral blood

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samples were consecutively collected in a period of 12 month. Blood samples (2–3 ml) were collected into EDTA-K3 tubes (Vacutainer; Becton Dickinson, Pont de Claix, France) and received in the laboratory within 1 hour after collection. The differential count and morphological analysis were performed by light microscopy on Leishman stained slides.

Surface Staining was done by Lyse–Wash method using 20 µl of antibodies added to 100 µl of whole blood in each tube.

The stained samples were treated with a lysing buffer to remove red blood cells while preserving the leukocytes. After centrifugation the pellet was re-suspended in 2 ml sheath fluid or PBS to stop lysis and the sample analyzed by 3-lasers, 7- color Flowcytometer and software program. Based on forward/side scatter (FSC/SSC) properties, a gate was set to exclude debris and fat cells. The leukemic population was controlled, but not determined, in the blast gate by CD45 staining. So partial overlapping with normal lymphocytes or monocytes was common.

We used CD 45 as a common marker for both lymphoid and myeloid lineage. CD5, CD7, CD10 and CD19 are positive for ALL among them CD7 and CD5 are positive for T-ALL and CD10 and CD 19 positive for B-ALL. CD117, CD 34, CD13 and CD33 are positive for AML case, and HLA-DR which is positive for both the cases. The eight parameters simultaneously collected were linear forward-angle light scatter (FSC), linear side scatter (SSC), log FITC, log PE, log Per Cp Cy 5.5, log PE Cy 7, log APC, log APC Cy 7. In order to keep the setting and histograms uniform during the studies, Flowcytometer was calibrated regularly with CST and 7 color setup beads.

List mode data on viable cells were analyzed by using the CD45/SSC Dot plot and four gates were setup: gate L for lymphocytes, M for monocytes, G for granulocytes and B for blast cells (Figure 1A & 2A).

The data of morphological analysis and differential count performed by light microscopy on Leishman stained slides were compared with four cell subpopulation data and arranged the results using bar diagrams (figure 3 and 4). We found the discrimination between the four cell subpopulations. These findings were not surprising because while CD45 expression was regularly lower on leukemic blasts than on normal lymphoid and monocytic cells, the FCS/SSC characteristics of these populations were overlapping.

**RESULTS:** CD45/SSC gating clearly separates the four cell categories of lymphocytes, monocytes, granulocytes and blast cells (Figure 1A & Figure 2A). Mature lymphocytes showed the highest CD45 fluorescence intensity and the lowest SSC signal (gate L). Mature monocytes expressed slightly lower but still high amounts of CD45 and were easily distinguished from lymphocytes by their higher SSC signal (gate M). Granulocytic lineage expressed highest SSC signal and lowest CD45 signal (gate G). Blasts cells had the lowest CD45 intensity and a low SSC; reflecting features of normal immature blood precursor cells (gate B). Erythrocytes and platelets, which did not express CD45 antigen, were not gated & considered as debris. The sum was not 100% since rare cells were found outside the gates.

Figure 1 (B1 to B4) shows the Blast cells positive for CD10, CD19, HLA-DR & negative for CD33, CD13, and CD7. From this result we could clearly conclude that it was a B-ALL case because all the lymphoid markers were positive except CD7, which was positive for T-ALL case.

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Figure 2 (B1 to B4) shows the Blast cells positive for CD13, CD33, CD117, HLA-DR & negative for CD10, CD19. From this result we could conclude that it was a case of AML because all the myeloid markers were positive.

In case of figure 3 and 4, we show the bar diagram of comparison between the light microscopic count and Flow cytometric count of four cell sub populations for ALL & AML respectively. From the above diagrams we find that the percentage of cell counts from the same peripheral blood sample differs in both the cell count procedure.

In 15 consecutive samples, the blood differential counts were compared with the results of the CD45/SSC gating procedure. A strong positive correlation was found for the four cell sub populations: Blast cells ( $r = 0.92$  in Figure 5), Lymphocytes ( $r = 0.75$ ), Granulocytes ( $r = 0.90$ ) and Monocytes ( $r = 0.90$ ).

**DISCUSSION:** In this study, we demonstrated the usefulness of a simple and reliable flowcytometry method to guide the Immunophenotyping. In ALL & AML cases, peripheral blood blast cells are frequently admixed to other cell lineages and the blast cells themselves are also heterogeneous. To avoid some of these problems, Ficoll density gradient centrifugation was frequently used to enrich samples in leukemic cells. Nevertheless, these steps have three major disadvantages:

1. A differential count of the leukemic sub-populations becomes unreliable and diverges from the differentials seen on smears;
2. Valuable information on the granulocytic cells is lost; and
3. The selective elimination of granulocytes from the sample also prevents their use as internal controls for staining mature myeloid cells.

Thus, we preferred to proceed with whole peripheral blood samples and to preserve all the nucleated cells in the sample by performing an erythrocyte lysis with the surface staining Lyse-Wash method - a technique, originally recommended for peripheral blood cell Immunophenotyping. Two limitations to the use of these methods have, however, been noted: (1) Immature erythroid cells are damaged and cannot be accurately analyzed. (2) Debris and fat cells need to be not gated by FSC/SSC.

We confirm earlier reports suggesting that the blast cell population can be clearly identified by virtue of their low antigen membrane CD45 antigen expression and their light scatter properties.<sup>[12-15]</sup> When analyzed by their FSC and SSC light properties, blast cells cannot be properly distinguished from lymphocytes and monocytes.<sup>[16,17]</sup>

In combination with CD45, SSC makes it possible to discriminate all major lineages in separate populations. In this work, we used APC Cy7-conjugated CD45 combined with FITC, Per CpCy 5.5, PE, PE Cy 7, and APC-conjugated antibodies in parallel samples to define the exact phenotypic expression of ALL & AML blast cells. One of the main findings of this paper is that this technique gives an excellent correlation with the light microscopic peripheral blood differential counts in smears( $r = 0.92$  in Figure 5).

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In conclusion, the expression of the 10 lymphoid and myeloid markers was an independent prognostic factor for outcome in patients with ALL and AML. The results from this study would be used to help developing diagnostic and treatment strategies that are based on the risk factors of the individual patient.

**ACKNOWLEDGEMENT:** This work has been supported and funded by the Department of Biotechnology (DBT) Project, Govt. of India, New Delhi.

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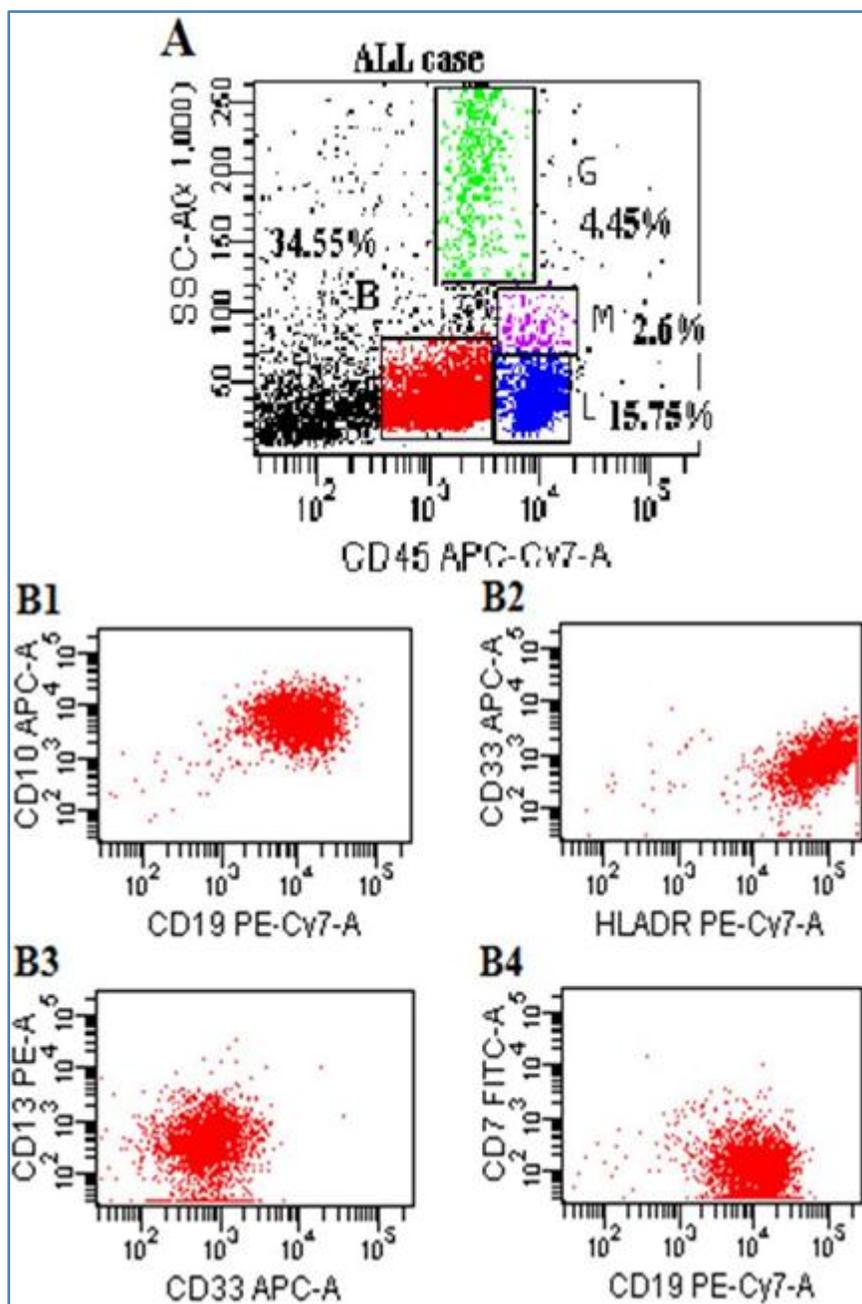
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<b>Antigen</b>	<b>Myeloblast</b>	<b>Maturing granules</b>	<b>Monocyte</b>	<b>B-Lymphoid</b>	<b>T-Lymphoid</b>
CD 5	–	–	–	–	+
CD 7	–	–	–	–	+
CD 10	–	+	–	Sub	–
CD 13	+	+	+	–	–
CD 19	–	–	–	+	–
CD 33	+	+	++	–	–
CD 34	+	–	–	Sub	–
CD 45	+	+	+	+	+
CD 117	+	–	–	–	–
HLADR	+	–	+	+	–

Table 1: Panel of monoclonal antibodies used in acute leukemia Immunophenotyping

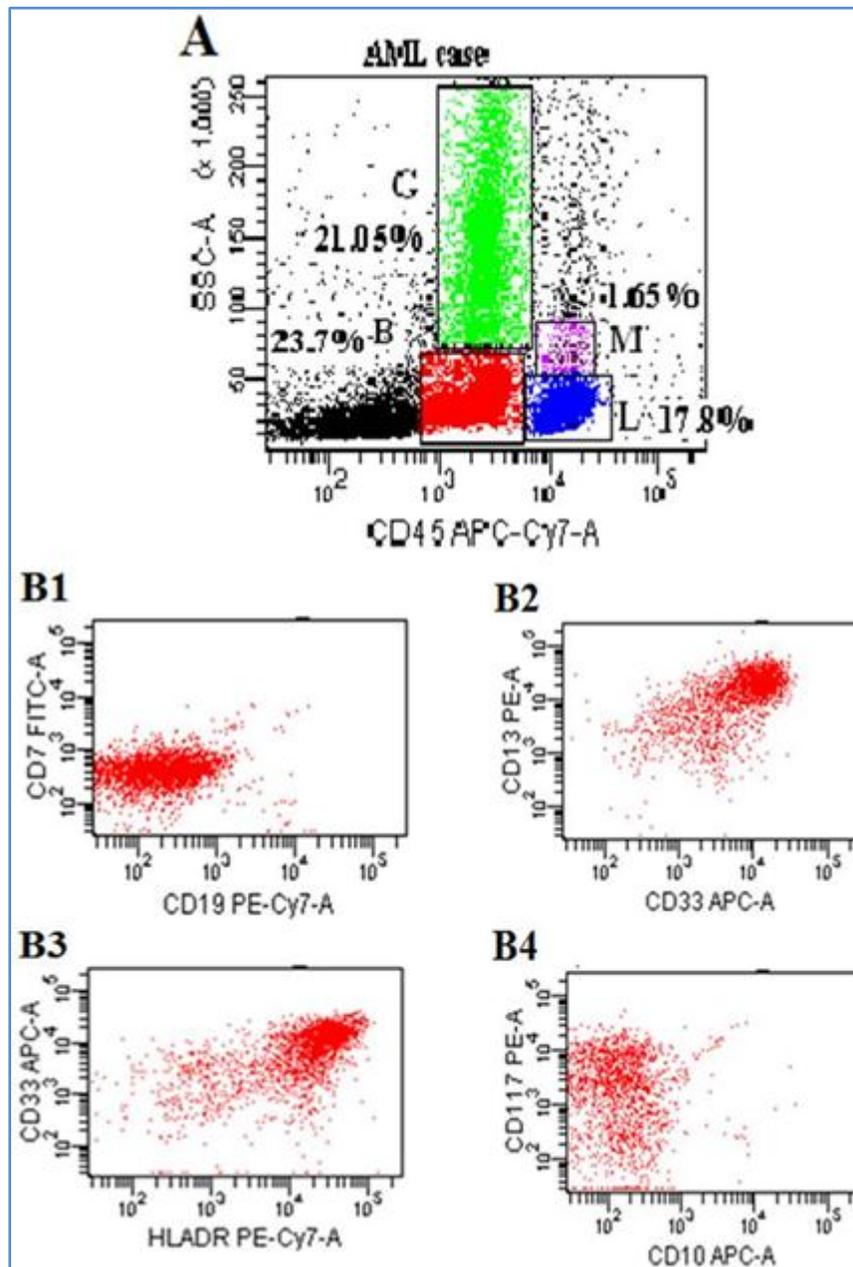
(- Absence of expression; + presence of expression; ++ bright expression)



**Fig. 1**

**Figure 1:** Flowcytometry Dot-plot of an Acute Lymphoid Leukemia (ALL)

- A. CD45/SSC with four gates: gate G including Granulocytes, gate M- Monocytes, gate L- Lymphocytes and gate B- Blast cells. Corresponding cell percentages are indicated.
- B. Dot plot of some CD expression in blast cells from the same sample.

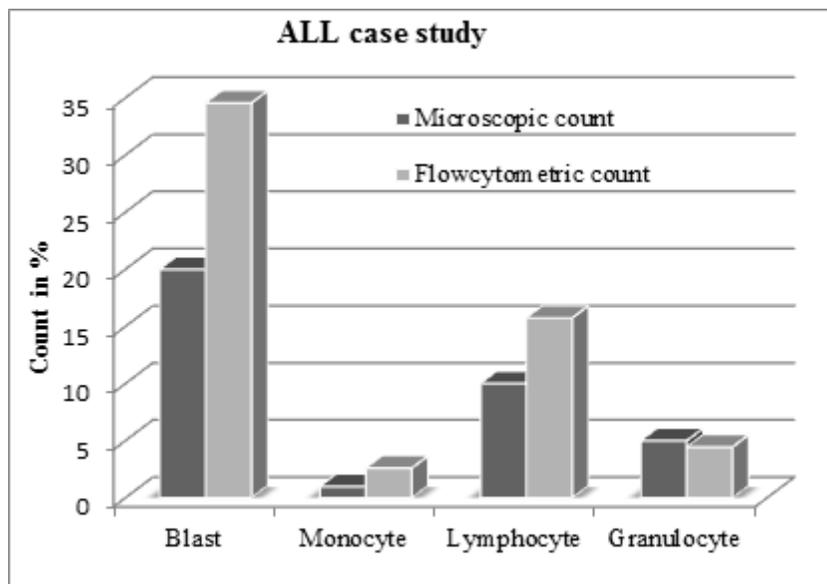


**Fig. 2**

**Figure 2:** Flowcytometry Dot-plot of Acute Myeloid Leukemia (AML).

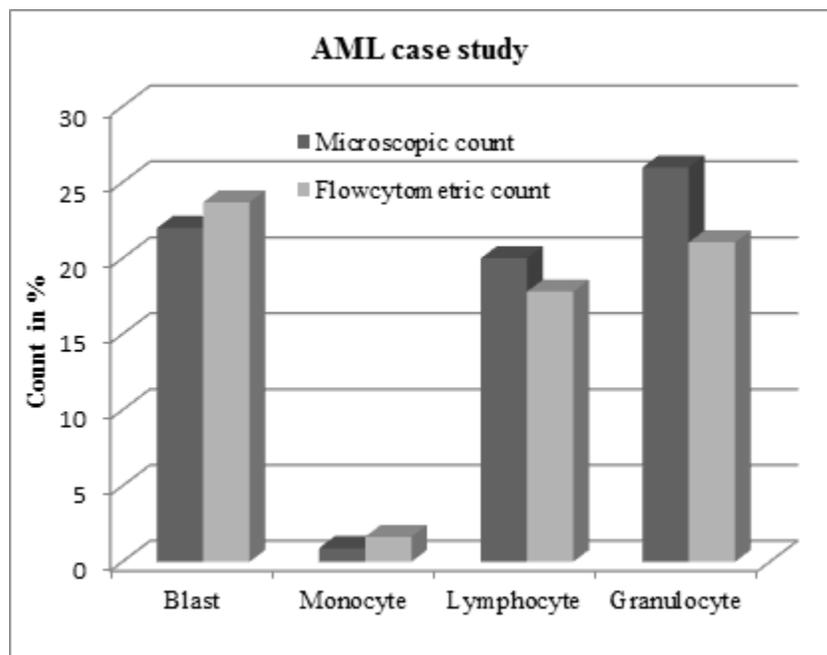
A. CD45/SSC with four gates: gate G including Granulocytes, gate M-Monocytes, gate L-Lymphocytes and gate B-Blast cells. Corresponding cell percentages are indicated.

B. Dot plot of some CD expression in blast cells from the same sample.



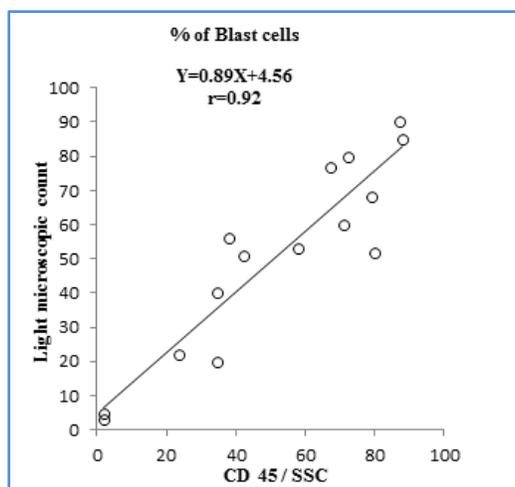
**Fig. 3**

**Figure 3:** Comparison between the light microscopic and Flowcytometric count in ALL case.



**Fig. 4**

**Figure 4:** Comparison between the light microscopic and Flowcytometric count in AML case.



**Fig. 5**

**Figure 5:** Correlations between a light microscopic differential count and the flowcytometric differential CD45/SSC gating for Blast cells evaluation.

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Date of Submission: 20/10/2014.  
Date of Peer Review: 21/10/2014.  
Date of Acceptance: 27/10/2014.  
Date of Publishing: 07/11/2014.