SIX COLOR NINE PART FLOW CYTOMETRIC DIFFERENTIAL

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INTRODUCTION:

Differentials distinguish between populations contained in a given sample. Current methods for differentials of blood samples are manual microscopy, hematology analyzers, and flow cytometry. Detection of abnormal populations in the blood is critical to the screening, diagnoses, and prognosis of many hematological diseases. The ability to do a white blood cell manual differential by current methods has several limiting factors, such as low reproducibility, difficulty with hypocellular samples, and limited classification.

Immunophenotyping by flow cytometry to cleanly identify leukocyte populations is not hindered by these limitations and has been used to identify the major lineages. Flow cytometry allows for an exponentially larger numbers of cells to be enumerated and analyzed. It facilitates the identification and classification of a variety of cellular subsets not detectable by current methods. It has been used to identify the major leukocyte lineages utilizing only 4 colors. This project works on validating a flow cytometric panel to be used for clinical implementation.

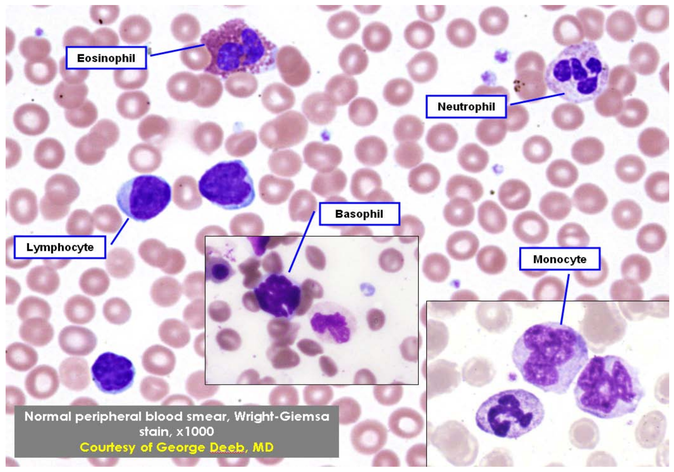


Figure 1. Classical representation of a normal blood smear. Populations of interest in this study are identified in the figure. In addition flow cytometry is able to differentiate Plasmacytiod Dendritic Cells and Lymphocytes subset (NK cells, B cells, T cells) by use of immunophenotyping..

OBJECTIVES:

- Identify 9 leukocyte populations in peripheral blood and bone marrow
- Expand on current flow cytometric methods (4 colors)
- Compare manual vs. flow cytometric differential
- Validate panel for clinical flow implementation

METHODS:

Human peripheral blood and bone marrow samples for flow cytometry analysis were collected in sodium heparin collection tubes. EDTA collection tubes were used for samples analyzed by Bone Marrow Lab medical technologist at Roswell Park Cancer Institute. Bone Marrow Lab prepared a smear of each of the samples and applied a Wright-Giemsa stain. A total of 10 healthy donor peripheral blood and 10 patient bone marrows were collected for this study. All flow cytometric data was ran on BD Fortessa cytometer, along with single tube for automated fluorochrome spillover compensation.

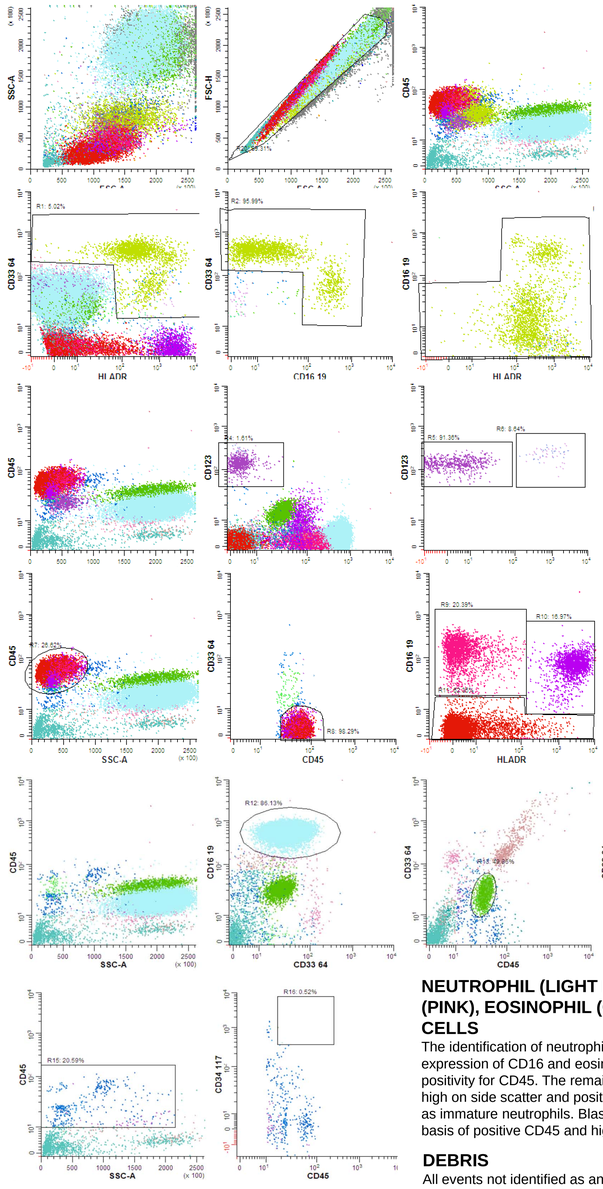
Fluorochrome-Antibody	Population
PE - CD123	Basophils, Plasmacytiod Dendritic cells
PC5 - CD33, CD64	Monocytes, Neutrophils
PECY7 - HLA-DR	Activated T cells, B Cells, Monocytes, PDC
APC - CD34, CD117	Blast Cells
V450 - CD16, CD19	NK cells, Neutrophils, Monocytes, PDC
V500 - CD45	Leukocytes

Table 1. Six color nine part flow cytometric differential panel; each antibody was optimized and validated.

BOOLEAN GATING STRATEGY:

Winlist Analysis Software (Verity)

Each population was identified using immuophenotypic analysis and subsequently excluded from further analysis using exclusionary gating techniques.



DOUBLET EXCLUSION
Aggregation happens in flow samples. To exclude aggregations that appear in our flow data, a gate around the singlet population was applied.

MONOCYTES (YELLOW-GREEN)
The monocytes were identified as positive for CD45, low on side scatter, positive for CD33, and heterogeneous for CD16 and HLADR.

BASOPHILS (PURPLE) + PLASMACYTIOD DENDRITIC CELLS (LIGHT PURPLE)
Following the gating and then exclusion of the monocyte population, basophils and PDCs were identified as CD123 positive and further separated with differing HLA-DR expression.

LYMPHOCYTES (T CELLS - RED, B CELLS – FUSCHIA, NK CELLS - PINK)
Lymphocytes were then identified as positive for CD45 and negative for CD33+64. The lymphocyte population was then separated with expression of CD16+19 and HLA-DR.

NEUTROPHIL (LIGHT BLUE), IMMATURE (PINK), EOSINOPHIL (GREEN), BLAST CELLS
The identification of neutrophils was based on positive expression of CD16 and eosinophil expression of positivity for CD45. The remaining population that was high on side scatter and positive for CD33 was identified as immature neutrophils. Blasts were identified on the basis of positive CD45 and high CD34+117 expression.

DEBRIS
All events not identified as any of the nine leukocyte population was labeled as debris. This value was subtracted from singlet population giving total population of leukocytes.

RESULTS:

Flow Cytometric Differential Data vs. Bone Marrow Differential Data

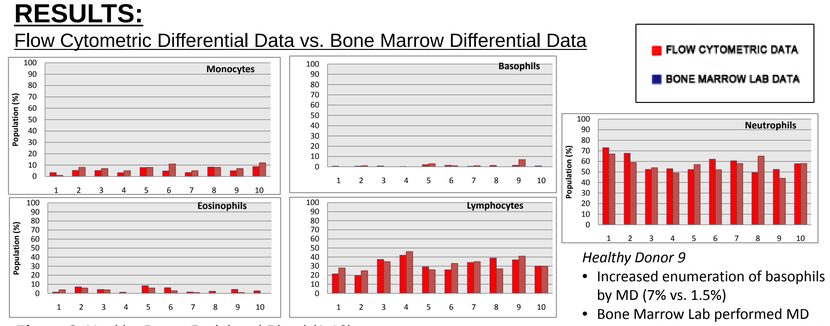
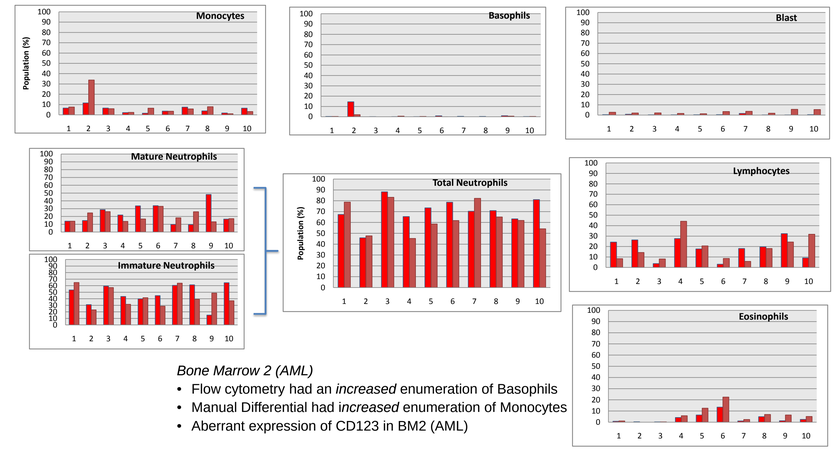


Figure 2. Healthy Donor Peripheral Blood (1-10)

Healthy Donor 9
- Increased enumeration of basophils by MD (7% vs. 1.5%)
- Bone Marrow Lab performed MD again and obtain value of 2 and 3%.



Bone Marrow 2 (AML)
- Flow cytometry had an *increased* enumeration of Basophils
- Manual Differential had *increased* enumeration of Monocytes
- Aberrant expression of CD123 in BM2 (AML)

Figure 3. Bone Marrow of patient with varying disease states (1-10)

CONCLUSIONS AND FURTHER STUDIES:

- Good correlation was observed between manual and flow cytometric differentials
- Flow cytometry has the advantage to identify more specific cell population based on antigen expression
- Increase sample size to increase statistical robustness
- Data analysis is ongoing to improve method of gating; delete redundancy
- Two additional colors that can be utilized
  - Additional differentiation of Erythroid precursor (CD71, CD235a)
  - Myeloid dendritic cell (CD11c)
  - Better define T cells (CD3)
  - Look into better markers for monocytes identification (CD14)
- We are getting closer towards implementation for clinical use

REFERENCES:

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