

# FOUR-PART DIFFERENTIAL LEUKOCYTE COUNT USING $\mu$ FLOW CYTOMETER

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## ABSTRACT

This paper reports the four-part differential leukocyte count (DLC) of human blood using a MEMS microflow ( $\mu$ flow) cytometer. It is achieved with a two-color laser-induced fluorescence (LIF) detection scheme. Four types of leukocytes including neutrophils, eosinophils, lymphocytes and monocytes are identified in blood samples, which are stained by fluorescein isothiocyanate (FITC) and propidium iodide (PI). The DLC results show good correlation with the count from a commercial hematology analyzer. The whole system is also implemented into a portable instrument for space application.

## INTRODUCTION

For NASA's long-term space missions, it's an emerging issue that how to handle health care of astronauts. During flights there's a lack of access to diagnosis capabilities. Differential leukocyte count (DLC), an important clinical capability providing diagnostics of infection, radiation exposure and other diseases astronauts might face [1], is one useful approach among the effects of solving this problem. Unfortunately, in-space DLC is still not available because commercial DLC analyzers are too bulky and not qualified for space use.

On the other hand, in-space DLC using  $\mu$ flow cytometers is attractive for the portable package size and the small sample volume. There are approaches measuring single cell impedance [2] or light scattering [3] to count leukocyte. However, so far only three-part differential (i.e., lymphocytes, monocytes, and neutrophils) has been reported using  $\mu$ flow cytometers [2], whereas the detection of eosinophils and basophils has not. Besides, the reported methods all require a significant dilution of blood sample.

This work uses the approach of fluorescent dye staining. Dye staining is a very common technique to identify biological tissues. It mainly targets intracellular features and there exist many types of dyes for leukocyte staining. The cost is also low, typically in the range of a few cents for each test.

However, one common problem of dye staining is the poor selectivity, which makes it challenging for

multi-part DLC. For instance, the nucleic acid dyes stain all nucleuses of different leukocytes with similar affinity. In our previous work [4], we encountered this problem with Acridine Orange dye and could only do a two-part DLC (i.e., lymphocytes and neutrophils).

In this work, we improve the approach by using the cocktail of two different dyes (i.e., FITC and PI) together with the laser-induced fluorescence (LIF) detection. This allows us to achieve the first four-part DLC (i.e., lymphocytes, monocytes, neutrophils and eosinophils) using MEMS  $\mu$ flow cytometers.

## DETECTION PRINCIPLE

As shown in Fig.1, a two-color LIF detection scheme is adopted for the  $\mu$ flow cytometer. A microfluidic channel is used as a flow cell, where dye-stained blood sample is measured for fluorescence emission [4]. The blood sample is illuminated by an excitation laser (488nm) at the sensing zone, and the emission is measured in two color ranges, green (510nm~560nm) and red (>590nm), which accommodate the emission peaks of FITC (520nm) and PI (617nm).

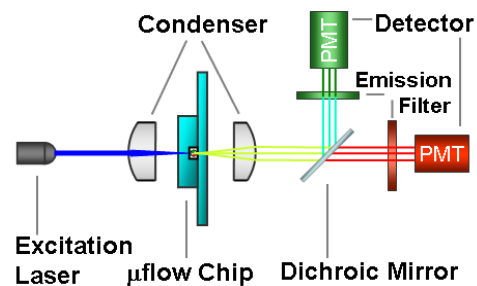


Figure 1: Schematic view of the two-color LIF detection used for leukocyte differential.

FITC is a protein staining dye which is mostly used as the fluorophore for antibody labeling. In permeable cells, it stains all the intracellular protein contents. It was reported that the FITC stains eosinophils with an affinity much stronger than other leukocytes [5]. In comparison, PI is a nucleic acid staining dye which has 20-fold enhanced fluorescence when binding to DNA/RNA. Only leukocytes have significant amount of nucleic acid contents among blood cells [6], so they can be selectively counted by PI fluorescence. The green fluorescence from protein staining by FITC and red fluorescence from nucleic acid staining by PI are

measured simultaneously to identify different types of leukocytes.

The volume of the detection zone (Fig.2) in the micro flow cell is designed to reduce cell coincidence which limits counting accuracy. In macro-size flow cytometer sheath flow is needed to reduce coincidence, where a sample is guided into narrow stream by hydrodynamic focusing. In  $\mu$ flow cytometer, the sample flow is constrained by the microfluidic channel dimension [4]. It can achieve a low coincidence rate without sheath flow, thus avoid using a large volume of buffer.

## $\mu$ FLOW CYTOMETER SYSTEM

Fig.2 shows the whole cytometer system we build and the  $\mu$ flow cartridge. The disposable cartridge is fabricated in a soft lithography process introduced before, where a PDMS block is bonded to a glass substrate to form the microfluidic channel [4]. The detection zone is  $28\mu\text{m}$  high and  $32\mu\text{m}$  wide, and its length is  $40\mu\text{m}$  defined by the size of excitation spot. This dimension is designed to be big enough to flow all blood cells through but small enough to reduce the coincidence error.

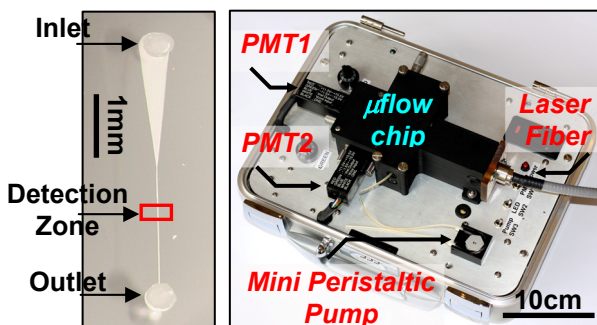


Figure 2: Top view of the fabricated  $\mu$ flow chip (left) and the portable  $\mu$ flow cytometer (right).

The system is built with off-shelf components except the flow cell. The excitation source is a 20mW, 488nm solid-state laser module. The optical components, such as the condensers, the dichroic mirror (HP580nm) and the emission filters (BP 510nm-560nm for green, HP 590nm for red) as shown in Fig.1, are assembled inside a black housing to be shielded from environmental light. Two photomultiplier tubes (PMTs) measure the green fluorescence and the red fluorescence separately but simultaneously. Blood sample is loaded by a pipette tip, and driven by a mini peristaltic pump for test.

The whole system, including the data acquisition module, is enclosed within an aluminum case ( $12'' \times 9'' \times 5''$ ). It could be powered by either the 110V AC or a 5V DC source, and the recorded data is read out by a

USB port. The portable system is suitable both for spaceflight applications and point-of-care applications.

## EXPERIMENTAL RESULTS

Human whole blood (with EDTA anticoagulant) were purchased from HemaCare Corp. (CA, USA) and tested within 24 hours after delivery. Blood samples were stained with the following recipe. First,  $5\mu\text{l}$  blood was mixed with  $5\mu\text{l}$  Cal-Lyse reagent (Invitrogen, USA) for 5 minutes to permeabilize all cells. The solution was then diluted by  $50\mu\text{l}$  distilled water to stop the permeabilization. Note that most erythrocytes were readily lysed at this point and the remaining ones were too diluted to interfere with the leukocytes count. After 3 minutes,  $8\mu\text{l}$  10X PBS buffer (Invitrogen) was added to stop the lysis. Then the sample was stained with  $6\mu\text{l}$  FITC solution ( $0.08\text{mg/ml}$  in water) and  $6\mu\text{l}$  PI solution ( $0.1\text{mg/ml}$  in water) for 10 minutes and finally loaded for test. The volumes were measured by adjustable-volume precision micropipettes.

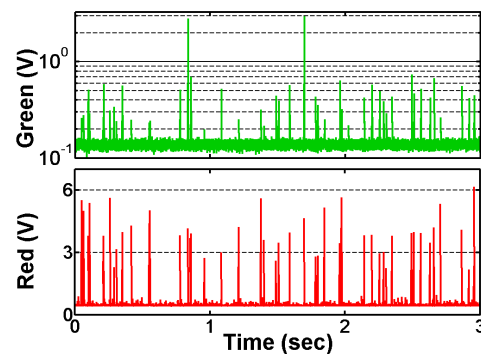


Figure 3: Recorded data of the measured fluorescence signals. Green and red are measured simultaneously.

Fig.3 shows a portion of the signals measured on the  $\mu$ flow cytometer. Each peak of the red fluorescence (PI) represents a leukocyte being counted, and the peak height indicates the amount of nucleic acid content. For each leukocyte event, the corresponding peak of the green fluorescence (FITC) measures the protein content. Some overlapped peaks caused by cell coincidence were observed during experiments, but the total number of overlapped peaks depicted a coincidence error of less than 1% for the given recipe.

Fig.4 is a typical scatter plot of 10,000 leukocyte events (green intensity versus red intensity), where each point represents one leukocyte. The scatter plot shows clearly four separated clusters. The cluster with highest green intensity is eosinophil, as they have strongest affinity to FITC among leukocytes [5]. The cluster with medium green intensity is neutrophil. Neutrophils contain many intracellular granules rich of protein, whereas mononuclear leukocytes

(lymphocytes and monocytes) don't. Therefore they have a relatively stronger FITC staining. This is consistent with the observations from reference [5]. The bottom left cluster is lymphocyte and the bottom right one is monocyte. Their green intensities are similar, but monocytes have more nucleic acid content thus higher red intensities than those of lymphocytes [6].

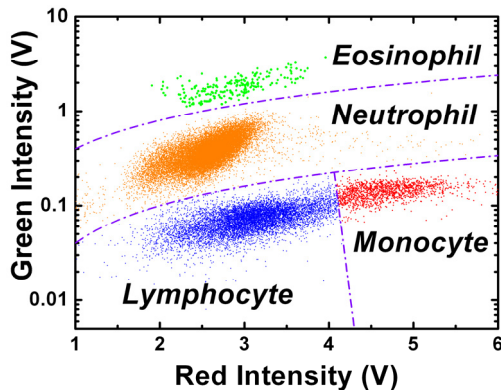


Figure 4: Scatter plot of recorded fluorescence intensities of leukocytes, green vs. red. 10,000 leukocytes were counted.

Reference	4-Part DLC	
6,000 cells/ml	5,800 cells/ml	Leukocyte

Figure 5: Summary of the 4-part DLC results. The reference is obtained from the same blood sample counted by a commercial hematology analyzer (i.e., Beckman Coulter LH750).

Fig.5 summarizes the results from a typical test including the concentration of total leukocyte and the percentages of each type (lymphocyte, monocyte, neutrophil and eosinophil), which constitute a complete four-part DLC report.

The accuracy test was carried out on blood samples from seven donors chosen randomly. Each sample was tested five times, which measured the average value and the maximum deviation. The results were then compared to the data measured from a commercial analyzer (Beckman Coulter LH750). Fig.6 shows the results of leukocytes concentration with a correlation coefficient  $r=0.98$  and a maximum error of 7% against reference values. Fig.7 shows the counted percentages of each type. The percentages of neutrophil, eosinophil and lymphocyte show good correlations ( $r \geq 0.97$ ) with

the reference values, whereas the monocyte correlation ( $r=0.76$ ) is not satisfactory and this is further studied in the DISCUSSION section below.

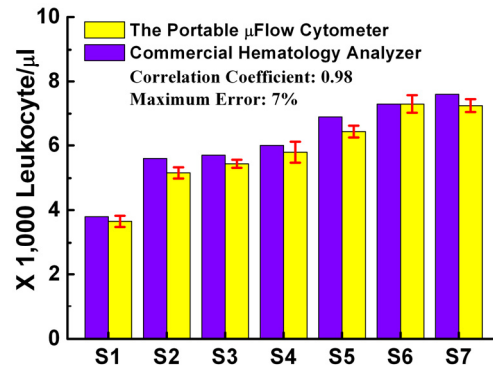


Figure 6: Results of leukocyte concentration compared to reference values. Correlation coefficient and maximum error are calculated. Error bars show maximum deviations from five repeated tests of the same blood sample.

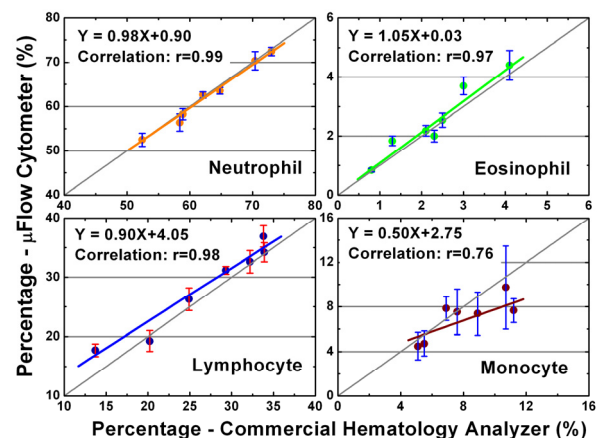


Figure 7: Correlation between the counted percentages and the reference values. For each leukocyte type, the linear regression and correlation coefficient are calculated. Error bars show the maximum deviations from five repeated tests.

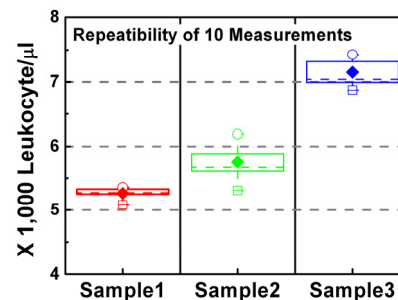


Figure 8: Leukocyte count repeatability for three samples in different concentration ranges. Each sample was used to repeat 10 tests.

The repeatability of counting leukocyte concentration was evaluated on three samples, and each sample was used to repeat 10 tests. Here the three samples were chosen to cover different concentrations ranges. Fig.8 shows the repeatability is reasonable with a maximum deviation less than 9%. Table 1 further illustrates the repeatability results on the percentage distribution from one sample.

Table 1: Test repeatability of the percentage distribution for one sample. The mean value (Mean) and the maximum deviation (MD) from ten repeated tests are listed. The reference values were obtained from the commercial analyzer.

	Tested		Reference
	Mean	MD	
Neutrophil (%)	56.3	1.9	58.4
Eosinophil (%)	2.1	0.2	2.3
Lymphocyte (%)	36.8	1.8	33.8
Monocyte (%)	4.8	1.2	5.5

## DISCUSSION

As mentioned above the monocyte percentage measurement shows a less ideal correlation ( $r=0.76$ ). This is a common and generic issue for monocyte count caused by the similarity between some large lymphocytes and monocytes. To study this issue, we did a separate experiment to verify the cluster identification of monocytes using an immunostaining specific for monocytes (i.e., anti-CD14-FITC from Invitrogen).

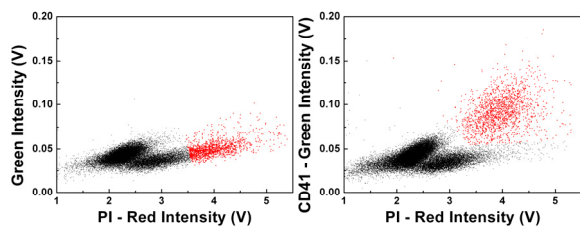


Figure 9: Identification of monocytes cluster. (a) The control result where monocytes (red dot) identified by PI fluorescence only. (b) The experimental result where monocytes identified by both PI fluorescence and green fluorescence from the anti-CD14 staining.

The control blood sample (Fig.9a) was stained with PI. The experimental sample (Fig.9b) was stained both with PI and the immunostaining. As expected, the green fluorescence of the immunostaining moves the monocyte cluster (marked red) up in the scatter plot (Fig.9b), so that the monocyte cluster is completely separated from the lymphocyte cluster. Fig.9b clearly shows that monocytes and lymphocytes do overlap in the zone of high PI intensity. This is consistent with the

4-part differential (Fig.4). As a result, the lymphocytes having strong red intensity are likely to be mistakenly counted as monocytes. Those cells were believed to be large lymphocytes [6]. Interestingly, the lymphocyte count itself is not affected by much because the total number of lymphocytes is much larger than that of the large lymphocytes.

## CONCLUSION

Four-part differential leukocyte count was demonstrated on a MEMS  $\mu$ flow cytometer. A two-color LIF detection scheme was used to identify lymphocytes, monocytes, neutrophils and eosinophils in human blood with fluorescent dyes FITC and PI staining. Blood samples from different donors are tested, and the results show good accuracy and repeatability.

## ACKNOWLEDGMENT

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## REFERENCES

- [1] B. Houwen, "The Differential Cell Count", Laboratory Hematology, vol. 7, pp. 89-100, 2001.
- [2] D. Holmes, D. Pettigrew, C. H. Reccius, J. D. Gwyer, C. Berkel, J. Holloway, D. E. Davies and H. Morgan, "Leukocyte Analysis and Differentiation Using High Speed Microfluidic Single Cell Impedance Cytometry", Lab Chip, vol. 9, pp. 2881-2889, 2009.
- [3] E. Altendorf, D. Zebert, M. Holl and P. Yager, "Differential Blood Cell Counts Obtained Using a Microchannel Based Flow Cytometer", Tech. Digest Transducers'97, Chicago, pp. 531-534.
- [4] W. Shi, S. Zheng, H. L. Kasdan, A. Fridge and Y.C. Tai, "Leukocyte Count and Two-Part Differential in Whole Blood Based on a Portable Microflow Cytometer", Tech. Digest Transducers'09., Denver, pp. 616-619.
- [5] E. Bedner, H. D. Halicka, W. Cheng, T. Salomon, A. Deptala, W. Gorczyca, M. R. Melamed and Z. Darzynkiewicz, "High Affinity Binding of Fluorescein Isothiocyanate to Eosinophils Detected by Laser Scanning Cytometry: A Potential Source of Error in Analysis of Blood Samples Utilizing Fluorescein-Conjugated Reagents in Flow Cytometry", Cytometry, vol. 36, pp. 77-82, 1999.
- [6] V. A. Loiko, G. I. Ruban, O. A. Gritsai, V. V. Berdnik, N. V. Goncharova, "Mononuclear Cells Morphology for Cells Discrimination by the Angular Structure of Scattered Light", Proc. 10th Conf. on Electromagnetic and Light Scattering by Non-spherical Particles, Bodrum, 2007, pp. 105-108.