

# Four-Parameter White Blood Cell Differential Counting Based on Light Scattering Measurements

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Received for publication May 4, 1987; accepted August 27, 1987

Measurement of the depolarized orthogonal light scattering in flow cytometry enables one to discriminate human eosinophilic granulocytes from neutrophilic granulocytes. We use this method to perform a four-parameter differential white blood cell analysis.

A simple flow cytometer was built equipped with a 5-mW helium neon laser that measures simultaneously four light scattering parameters. Lymphocytes, monocytes, and granulocytes were identified by simultaneously measuring the light scattering intensity at angles between 1.0° and 2.6° and angles between 3.0° and 11.0°. Eosinophilic granulocytes were distinguished from neutrophilic granulocytes by simultaneous measurement of the orthogonal and depolarized orthogonal

light scattering.

Comparison of a white blood cell differentiation of 45 donors obtained by the Technicon H-6000 and our instrument revealed good correlations. The correlation coefficients ( $r^2$ ) found were: 0.99 for lymphocytes, 0.76 for monocytes, 0.99 for neutrophilic granulocytes, and 0.98 for eosinophilic granulocytes. The results demonstrate that reliable white blood cell differentiation of the four most clinically relevant leukocytes can be obtained by measurement of light scattering properties of unstained leukocytes.

**Key terms:** Differential leukocyte counting, flow cytometry, eosinophils, automation, CBC

White blood cell differentiation is routinely performed in a variety of ways. In manual differential counting, five different leukocytes are commonly distinguished. From the early 1960s, attempts have been made to automate differential leukocyte counting. This has resulted in a variety of commercial blood cell analyzers. Cells in flow systems are detected either electronically (aperture impedance) or optically. The only flow system that can perform reliable five-part differential white blood cell counting is produced by Technicon (H-6000, HI). But in order to achieve this, several cytochemical staining steps of the leukocytes are required. Therefore, these systems are relatively complicated. Presently available flow systems of unstained leukocytes can only distinguish three leukocytes populations, i.e., granulocytes, monocytes, and lymphocytes.

Recently we have introduced depolarized orthogonal light scattering as a new parameter in flow cytometry (2). By measurement of this parameter simultaneously with the orthogonal light scattering, eosinophilic granulocytes can be clearly distinguished from neutrophilic granulocytes (2.5). We thus extended the differential counting of unstained leukocytes to four clearly distinguishable leukocyte populations. In this study we com-

pared the differential white blood cell counting of the Technicon H-6000 with the results obtained with our instrument.

## MATERIALS AND METHODS

### Sample Preparation

Human blood was collected by venipuncture into vacutainer tubes containing EDTA as anticoagulant [Venject, EDTA (K<sub>3</sub>), Terumo Europe NV]. Venous blood, 10  $\mu$ l, was added to 200  $\mu$ l lysing reagent (Lysright, Ortho Diagn. Syst., Aubervilliers, France). Lysright is a slightly alkaline aqueous solution containing 2-butoxy-ethanol. The suspension was gently shaken for 30 s after which 200  $\mu$ l of phosphate-buffered saline was added. The sample was then ready for measurement. The buffer was added in order to minimize changes of leukocytes by the lysing reagent during measurement. Measurements could be performed up to 30 min after lysing without disturbing the light scattering properties of the leukocytes.

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### Flow Cytometer

The optical measurements were performed in a quartz flow cell with a  $250 \times 250\text{-}\mu\text{m}^2$  flow channel designed by us and produced by Hellma (Hellma GmbH & Co., Mullheim/Baden, Federal Republic of Germany). A stable flow was achieved by pressurizing the sheath and sample flow. As light source, a 5-mW helium neon laser model 120S (Spectra Physics, San Jose, CA) was used. The laser was polarized in the direction of the sample stream. The laser was focused on the cell stream by means of a spherical lens (focal length 100 mm). The forward scattered light was collected with a spherical lens (focal length 40 mm) provided with a circular beam stop. Two different narrow-angle forward scatter cones were obtained by using a mirror with a central hole placed at  $45^\circ$  to the laser beam. The first cone was reflected by the mirror. It contained light scattered at angles between  $3.0^\circ$  and  $11^\circ$  to the incoming laser beam. The second cone passed through the central hole in the mirror. It contained light scattered at angles between  $1.0^\circ$  and  $2.6^\circ$  to the incoming laser beam. The scattered light was detected with photodiodes (model Pin 10 D, United Detector Technology). Orthogonal light scattering was collected with a Leitz microscope objective (H32, NA 0.6 collecting angles between  $115^\circ$  and  $65^\circ$ ) and was imaged on a diaphragm. A small portion of the orthogonally scattered light was directed to the first photomultiplier R928 (Hamamatsu), which measures total orthogonal light scattering, with a glass plate placed at  $45^\circ$  to the light path. The transmitted portion of the light went to a second photomultiplier (Hamamatsu), which was provided with a polaroid filter HN7 (Melles Griot, Irvine, CA). This filter was oriented so as to absorb scattered light polarized parallel to the sample stream. The scattered light detected with this photomultiplier will be referred to as depolarized orthogonal light scattering. The four light scattering intensities were digitized and stored in memory by DMA (Direct Memory Access) for each separate cell. For each measurement, 8,192 cells were analyzed. The data were collected in list mode with a personal computer-based data acquisition system developed in our laboratory (3). This system consists of a home-made analogue signal processing unit, an RTI 800 multi function Input/Output Board (Analog Devices, Northwood, MA), and an Olivetti M-24 personal computer (640 KByte Olivetti & Co., Ivrea, Italy).

White blood cell differentials obtained by the instrument described in this section were compared with a commercial blood cell analyzer, the H-6000 (Technicon Instrument Corp., Tarrytown, NY). The performance of this instrument has been described in detail elsewhere (1).

### RESULTS

Simultaneous measurements of four light scattering parameters were performed as described in Materials and Methods. Two forward light scattering parameters were determined from the scattered light collected at angles between  $1.0^\circ$  and  $2.6^\circ$  and between  $3^\circ$  and  $11^\circ$ ,

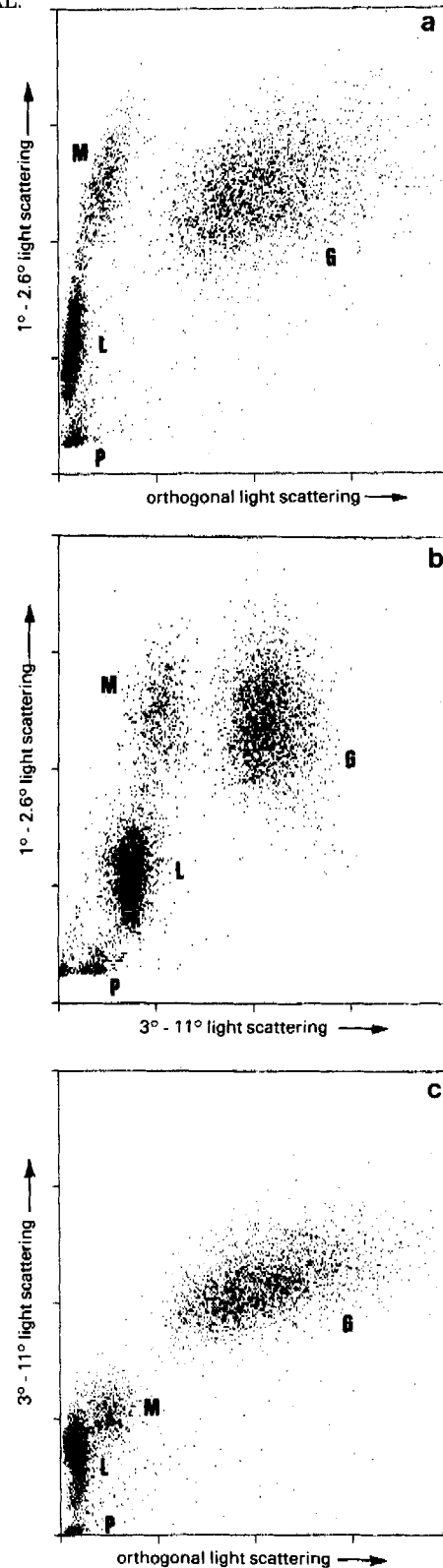


FIG. 1. Scatter plots of light scattering measurements of a typical leukocyte suspension. a)  $1\text{-}2.6^\circ$  light scattering vs. orthogonal light scatter. b)  $1\text{-}2.6^\circ$  light scattering vs.  $3\text{-}11^\circ$  light scattering. c)  $3\text{-}11^\circ$  light scattering vs. orthogonal light scattering. Note that the information obtained from the three light scattering parameters is different. L, lymphocytes; M, monocytes; G, granulocytes; P, platelets, red cell ghosts, and debris.

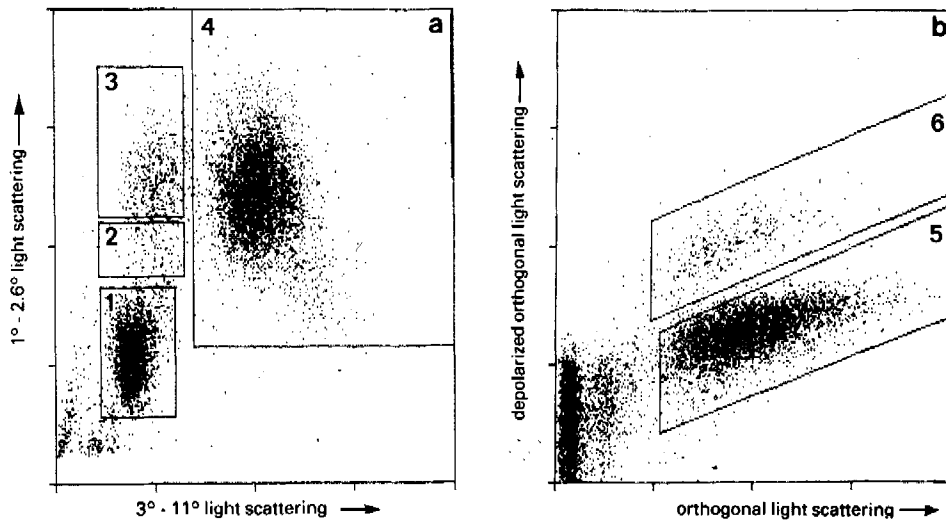


FIG. 2. Scatter plots of light scattering measurements of a typical leukocyte suspension. a) 1-2.6° light scattering vs. 3-11° light scattering. b) Depolarized orthogonal light scattering (log scale) vs. orthogonal light scattering. The gates that were used in all measurements for

determination of the percentage of the different leukocytes are indicated. Gate 1: lymphocytes; gate 3: monocytes; gate 4: granulocytes; gate 5: neutrophilic granulocytes; gate 6: eosinophilic granulocytes. In gate 2, basophilic granulocytes, monocytes, and lymphocytes are found.

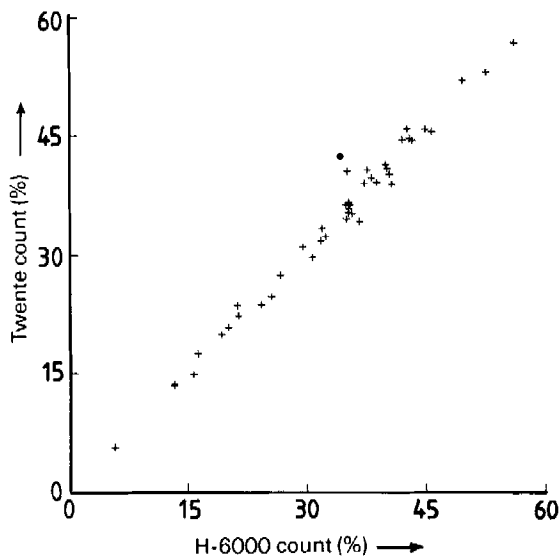


FIG. 3. Percentage of lymphocytes in peripheral blood as determined with the Technicon H-6000 (H-6000-count) and our flow cytometer (Twente count) for 45 donors. Correlation coefficient:  $r_2 = 0.99$ . The best linear fit has a slope of 1.02.

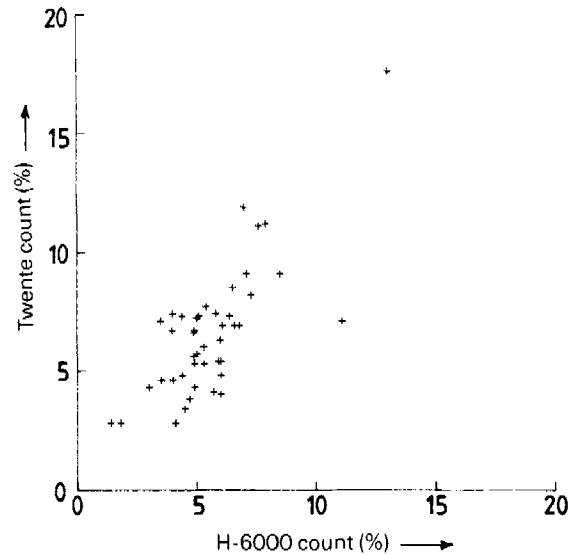


FIG. 4. Percentage of monocytes in peripheral blood as determined with the Technicon H-6000 and our flow cytometer (Twente) for 45 donors. Correlation coefficient:  $r^2 = 0.77$ . The best linear fit has a slope of 1.01.

and two orthogonal light scattering parameters were determined from the orthogonal light scattering between 65° and 115° and the depolarized orthogonal light scattering between 65° and 115°.

The use of two narrow-angle scatter parameters appeared to be useful for the separation of the different leukocyte populations. This is illustrated in Figure 1. Figure 1a shows a scatter plot of the orthogonal light scattering vs. the 1.0-2.6° light scattering. These scatter

plots are the ones most commonly used in flow cytometry. The discrimination between lymphocytes, monocytes, and granulocytes can be seen. When the 1-2.6° light scattering is plotted against the 3-11° light scattering, the separation of these populations is at least as good (Fig. 1b). Note that both narrow-angle scatter parameters contribute to the improved separation of lymphocytes from platelets and red cell ghosts (P). In Figure 1c, a scatter plot is shown of the orthogonal light

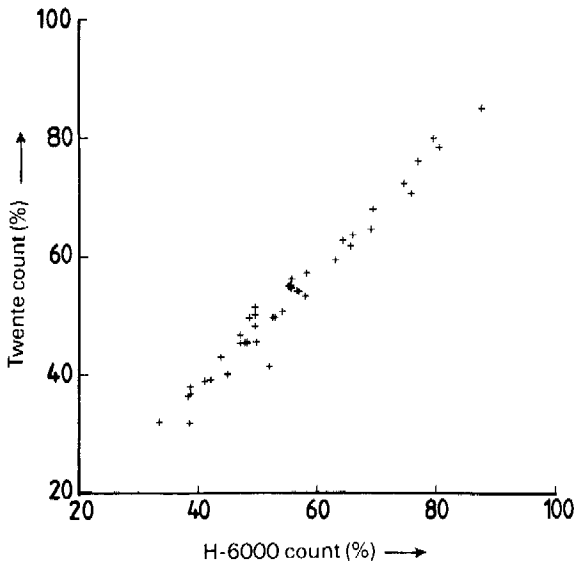


FIG. 5. Percentage of neutrophilic granulocytes in peripheral blood determined with the Technicon H-6000 and our flow cytometer (Twente) for 45 donors. Correlation coefficient:  $r^2 = 0.99$ . The best linear fit has a slope of 1.00.

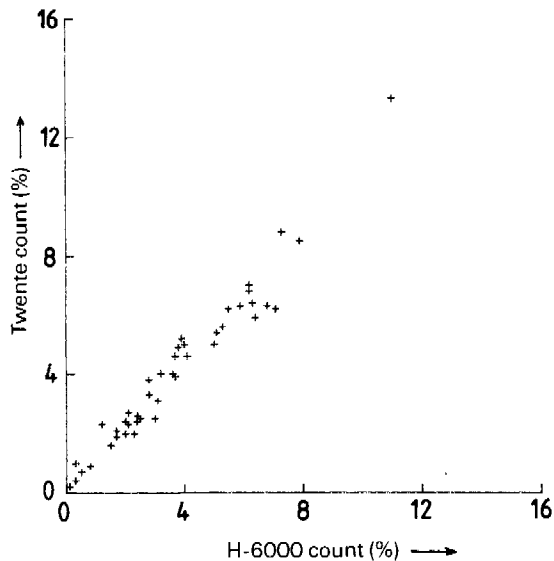


FIG. 6. Percentage of eosinophilic granulocytes in peripheral blood determined with the Technicon H-6000 and our flow cytometer (Twente) for 45 donors. Correlation coefficient:  $r^2 = 0.97$ . The best linear fit has a slope of 1.07.

scattering versus the  $3^\circ$ – $11^\circ$  light scattering; the figure illustrates that both scatter parameters yield different information. Of additional importance is that the orthogonal light scattering does not help in the discrimination of lymphocytes from platelets and red cell ghosts, in contrast to the  $3$ – $11^\circ$  scattering. The use of  $3$ – $11^\circ$  light scatter also helped to identify cells in patients with haematological disorders such as chronic myeloid leukemia and infectious mononucleosis (data not shown).

For comparison with results obtained on a Technicon H-6000, we used the  $1$ – $2.6^\circ$  scattering vs. the  $3$ – $11^\circ$  scattering for the discrimination between lymphocytes, monocytes, and granulocytes. In Figure 2 two scatter plots are shown of light scattering measurements of a typical unstained leukocyte sample. In Figure 2a the  $1$ – $2.6^\circ$  light scattering is plotted vs. the  $3$ – $11^\circ$  light scattering. Four gates are indicated in the figure. It appeared that the majority of the lymphocytes fall within gate 1, monocytes in gate 3, and granulocytes in gate 4. As can be seen in the figure, gate 2 was not placed on a clearly distinguishable population. However, we have observed that in cases with a high basophilic granulocyte count the majority of the basophilic granulocytes fell within this gate. In Figure 2b the orthogonal light scattering is plotted vs. the depolarized orthogonal light scattering (log scale) for the same measurement. The gate on the leukocyte population with a relatively low depolarized orthogonal light scattering signal (gate 5) contains the majority of the neutrophilic granulocytes, whereas the gate on the leukocyte population with a relatively high depolarized orthogonal light scattering signal (gate 6) contains the majority of the eosinophilic granulocytes.

For comparison of the leukocyte differentiation obtained with the Technicon H-6000 and our apparatus, we have used the gate setting as illustrated in Figure 2. It was not necessary to reset the gates for different measurements. The leukocyte differentiations of 45 healthy donors obtained with the two instruments were compared. The correlations of the results obtained for lymphocytes is shown in Figure 3 [correlation coefficient  $r^2 = 0.99$ , slope best linear fit (slf) 1.02]; for monocytes in Figure 4 ( $r^2 = 0.99$ ; slf 1.01); for neutrophilic granulocytes in Figure 5 ( $r^2 = 0.99$ ; slf 1.00); and for eosinophilic granulocytes in Figure 6 ( $r^2 = 0.98$ , slf 1.07). The cell count obtained from the gate in which the basophilic granulocytes fell did not correlate with the basophilic granulocyte count obtained with the H-6000. The number of basophilic granulocytes was overestimated in our system. The very low frequency of basophilic granulocytes (less than 2%; H-6000 count) in these experiments makes the role of a few monocytes and lymphocytes within our basophilic granulocyte gate 2 very important.

## DISCUSSION

In flow cytometry light scattering measurements are routinely used to select different cell populations. Salzman et al. have shown that by measurement of the orthogonal and forward light scattering of unstained leukocytes, lymphocytes, monocytes, and granulocytes can be distinguished (4). Recently we have shown that cytotoxic lymphocytes can be distinguished from regulatory and B lymphocytes because of a different intensity of the orthogonal light scattering signal (6). In addition, we have introduced depolarized orthogonal light scattering as a powerful new parameter with which neutrophilic granulocytes could be distinguished from eosinophilic granulocytes (2,5).

In this study we have used a simple flow cytometer that enables us to distinguish lymphocytes, monocytes, and eosinophilic and neutrophilic granulocytes. It uses a low-power helium neon laser as light source and measures simultaneously four light scatter parameters.

Data were collected in list mode using a relatively inexpensive personal computer. With two narrow angle scatter parameters, a clear separation between lymphocytes, monocytes, and granulocytes was obtained. We found that by using the 3–11° light scattering, better results were obtained than by using the orthogonal light scattering as is normally done. In this way a better discrimination between lymphocytes and platelets, red cell ghosts, and debris was obtained. In addition, it has to be emphasized that in doing so one is able to construct a very simple three-part white blood cell analyzer that does not need orthogonal detection optics or photomultipliers.

Using a low-power helium neon laser, a clear separation of neutrophilic and eosinophilic granulocytes was obtained by measurement of the orthogonal and depolarized orthogonal light scattering. For a four-part white blood cell differentiation, gates could be set on clearly distinguishable populations.

We have seen that in rare cases with exceptional high basophil counts, the basophilic granulocytes fall in region 2, indicated in Figure 2. Our results show, however, that for normal healthy individuals, discrimination by this gate is not sufficient to obtain reliable basophil counts. We are currently investigating in our laboratory different light scattering parameters by which low numbers of unstained basophilic granulocytes hopefully can be distinguished.

Four-part leukocyte differentiation of 45 donors performed on our light scatter-based flow cytometer was compared with the results obtained on a Technicon H-6000 blood cell analyzer. The latter instrument is described as a reliable instrument for five part differential white blood cell counting (1). It uses, however, additional staining procedures of the leukocytes to achieve this

goal, which makes this flow cytometer quite complicated and expensive. Comparison of both instruments revealed good correlations between percentages of lymphocytes, neutrophilic granulocytes, and eosinophilic granulocytes. The observed correlation for monocytes was less good ( $r^2 = 0.76$ ). A similar poor correlation is observed between the Technicon H6000 and manual count,  $r^2 = 0.72$  (1). According to these results, we have demonstrated the feasibility of a four-part differential leukocyte counter using a flow cytometer that measures light scattering properties of unstained leukocytes. Further studies on clinical samples are needed to investigate the potential use of this method to detect abnormal cells in different diseases.

#### ACKNOWLEDGMENTS

We thank the technicians of the haematological laboratory of Ziekenzorg Hospital, Enschede, The Netherlands for sample analysis on the Technicon H-6000.

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