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A New Method for Fast Blood Cell Counting and Partial Differentiation by Flow Cytometry*

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Summary. A new blood counting method by flow cytometry is described which determines absolute counts and relative proportions of erythrocytes, reticulocytes, thrombocytes, lymphocytes and granulocytes from one sample of saline diluted human or animal blood. Staining time is 2 to 5 min and measuring time between 1 and 2 additional minutes. Measured simultaneously are the electrical cell volume, the green and optionally also the red fluorescence of the transmembrane potential sensitive dye 3,3-dihexyloxacarbocyanine DiOC6(3) and the RNA/DNA stain acridine orange (AO). Work is under way to fully automate staining, measurement and data evaluation. The use of stains by which blood cell counting and biochemical analysis can be combined offers new possibilities for routine blood cell counting without requirement for additional time. The potential of such stains is that pathologic cell conditions which are not, or not yet reflected in the cell count may be earlier detectable by biochemical stains.

Key words: Flow-cytometry – Blood cell count

Introduction

Blood cell counting and blood cell differentials are routine procedures which in the case of erythrocytes, leukocytes and thrombocytes are in many instances performed by electronic counters operated manually or automatically. The electronic instruments often do not count the different cell types in one diluted blood sample but count on several dilutions of the original blood which increases the technical requirements. Leukocyte differentials and reticulocytes are mostly microscopically determined in separate smears after adequate staining of the cells. Microscopical evaluations are time consuming and tedious especially in the case of reticulocytes.

We have developped a new staining method which avoids several of these inconvenients and gives besides counting, additional information on the biochemical state of different blood cell types. A single sample of diluted blood is measured in a flow-

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cytometer after short staining with fluorescent dyes at room temperature. Absolute counts and relative proportions of erythrocytes, reticulocytes, thrombocytes, lymphocytes and granulocytes, as well as, the functional state of the various blood cell types are obtained.

Material and Methods

500 µl of 1/250 saline (+ 10 mM TRIS/HCl pH 7.35, TBS) diluted blood were stained at room temperature during 5 min after addition of 5 µl dye cocktail. The dye cocktail is composed of the transmembrane potential sensitive dye 3,3-dihexyloxacarbocyanine (DiOC 6 (3), Eastman Kodak, Rochester, N.Y) [1] at a concentration of 10 µg/ml, acridine orange (AO, Serva, Heidelberg, FRG) for RNA/DNA staining at a concentration of 400 µg/ml. The dye cocktail additionally contained 6 µm monosized, NH_2-group bearing, fluorescein-isothiocyanate stained, porous latex particles [2] at a concentration of 1 or 2×10^7 particles/ml. The solvent of the cocktail consisted either of dimethyl-sulfoxide or dimethyl-formamide. The electrical cell volume and the fluorescence of each cell or particle were measured simultaneously for 1 to 2 min at a speed of 1000–2000 cells/s in a Fluvo-Metricell flow cytometer [3] using TBS buffer as suspending medium and a 50 µm cylindrical orifice of 60 µm length at an electrical current of 0.23 mA through the orifice. The particle beam was hydrodynamically focused in the center of the orifice. Cellular fluorescence was excited between 400 to 500 nm with a HBO-100 high pressure mercury arc lamp and collected between 500 to 700 nm with a photomultiplier tube. The maximum amplitude of each signal was amplified by 2.5 decade logarithmic amplifiers, collected in a 64×64 matrix of a multichannel analyzer and displayed with a 3 decade logarithmic amplitude scale which was divided into ten linear steps by contour lines to display and calculate the various cell and particle clusters with programs developed earlier [4].

For reasons of comparison with other current methods, the erythrocytes were counted from aliquots of all blood samples in a Coulter model A counter (Coulter, Hialeeh, Florida, USA) with a 70 µm orifice at a dilution of 1 : 50,000 in TBS buffer. The thrombocytes were counted manually after 1/100 dilution of blood with a 1% ammonium-oxalate solution [5]. The blood reticulocytes were stained in suspension after 1+1 dilution by volume with a 10 mg/ml brillant-cresyl-blue solution in TBS for 1 h at room temperature and counted microscopically after preparation of a smear (500 cells per count).

Results

A typical histogram of a flow cytometric measurement of DiOC 6/AO stained blood cells from a healthy human adult (Fig. 1) displays four separate clusters which are: the thrombocytes, the calibration particles, the lymphocytes and the granulocytes. In addition the fused erythrocyte/reticulocyte cluster and a background cluster are visible. The clusters of thrombocytes, lymphocytes, granulocytes and standardizing particles can be simultaneously projected onto the cell volume axis (Fig. 2a, b) for better visual evaluation. Similarly volume distribution curves of the erythrocytes and reticulocytes can be plotted (not shown) from Fig. 1 by projection.

The cells of Fig. 1 were simultaneously stained with DiOC 6 and AO in order to separate the small thrombocytes well from the background. All cell clusters were, however, also visible with DiOC 6 or AO staining alone. DiOC 6 stains well thrombocytes, lymphocytes and granulocytes but to a lesser degree reticulocytes and erythrocytes. Approximately 60% of the stain in thrombocytes, lymphocytes and granulocytes is due to DiOC 6 under the staining conditions used. AO alone stains well

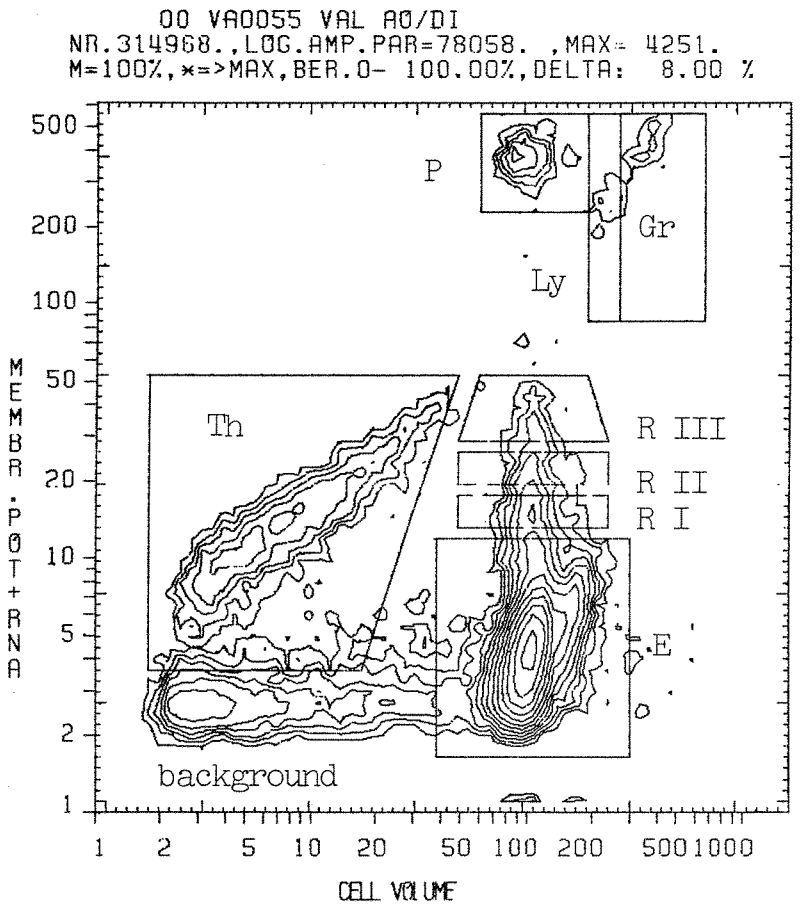

Fig. 1. Cell volume versus fluorescence plot of AO and DiOC 6 (3) stained human peripheral blood cells. The cell clusters (*Th* = thrombocytes, *P* = calibration particles, *Ly* = lymphocytes, *Gr* = granulocytes, *E* = erythrocytes, *RI, RII, RIII* = mature, intermediate and immature reticulocytes) are delimited by lines which define the areas for computer evaluation. The results of the evaluation are given in Table 1. A total of 78058 cells were counted during the measurement and each cell was classified according to the logarithmic height of the cell volume and fluorescence signals into a 64×64 channel matrix (linear abscissa and ordinate scales). The amplitude scale comprises 3 logarithmic decades and is divided in ten linear steps for each of which a contour line is plotted. The contour lines of the cell clusters were calculated from the channel contents to improve the visual impression of the graph. The highest contour line corresponds to a channel content of 4251 cells, the lowest line corresponds to a channel content of 2 cells. Channels with a content of one cell are not contoured. The logarithmic fluorescence scale on the ordinate is expressed in relative fluorescence units. One volume unit on the logarithmic abscissa scale corresponds to 0.92 fl. The mean volumes of the various cell types are given in Table 1.

the reticulocytes, lymphocytes and granulocytes but thrombocytes and erythrocytes are only weakly stained. The total fluorescence of each cell in the various cell clusters was divided by the cell volume in order to obtain the relative intracellular dye concentration (RDC). The RDC is a composite measure of the transmembrane potential, of the RNA concentration, and in lymphocytes and granulocytes also of the DNA. The RDC is a typical constant for each cell type. Changes of the RDC represent an alteration of the functional state of cells with regard to transmembrane potential or RNA but may also indicate DNA aneuploidies in the case of lymphocytes and granulo-

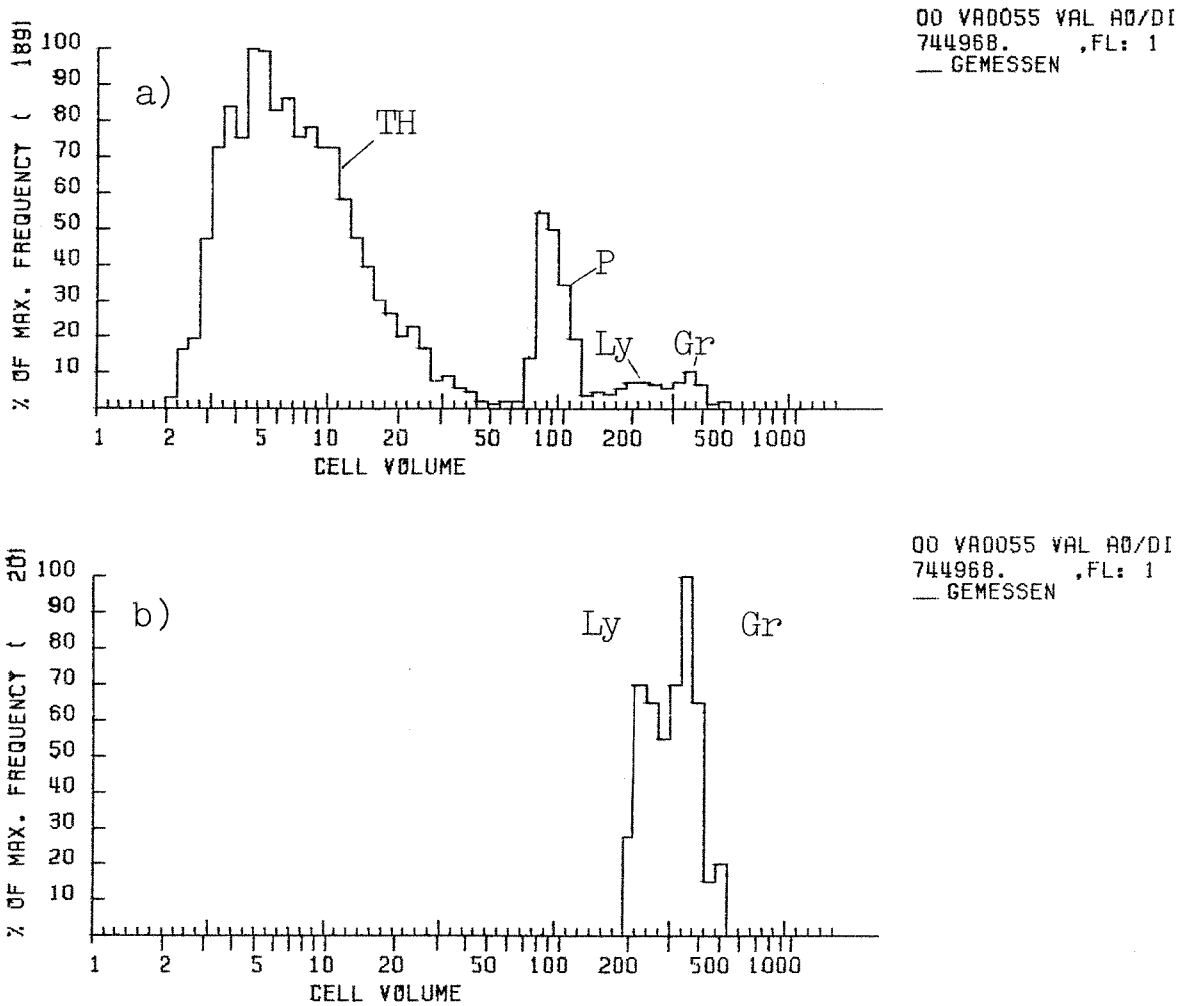

Fig. 2. Volume distribution curve of: (a) thrombocytes (*TH*), calibration particles (*P*), lymphocytes (*Ly*) and granulocytes (*Gr*) together, and (b) of lymphocytes and granulocytes alone. The curves were obtained by projecting the respective cell clusters of Fig. 1 onto the cell volume axis

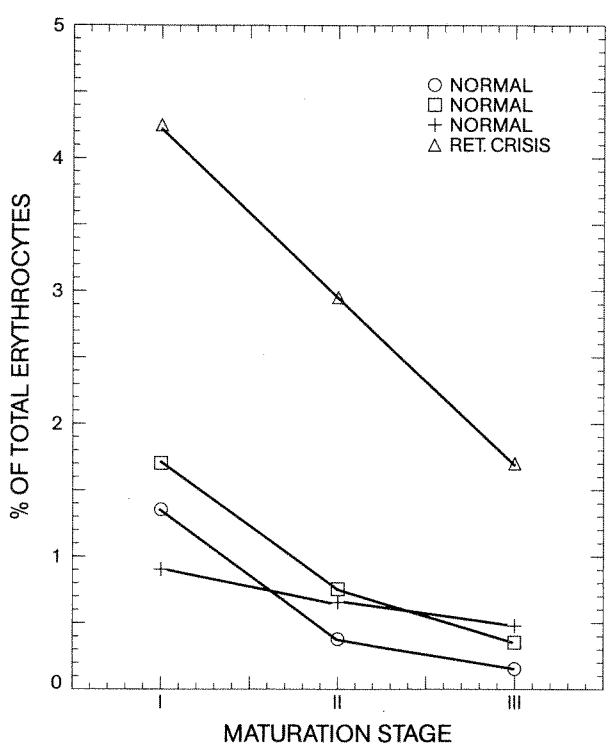

Fig. 3. Reticulocyte maturation pattern of three healthy human adults and one patient during a reticulocyte crisis after severe bleeding. The data were collected from histograms similar to Fig. 1 within the areas RI, RII and RIII which delimit mature, intermediate and immature reticulocytes

Table 1. Calculated cell concentrations and cell parameters of blood stain in Fig. 1

	Thrombo-cytes	Erytro-cytes	Reticulocytes RI	RII	RIII	Total retic.	Lym-pho-cytes	Gra-nulo-cytes	Leu-ko-cytes
Cell concentration	2.51x10^5/µl	5.21x10^6/µl	0.82%	0.36%	0.17%	1.35%	2870/µl	3630/µl	6500/µl
Reference methods	2.73	5.15	—	—	—	1.50	—	—	6700
Mean cell volume (fl)	7.37	92.3	95.4	97.2	94.4	95.1	238	359	—
CVD (%)	71.9	15.7	—	—	—	17.2	13.2	16.7	—
Mean RDC	1.01	.021	.076	.104	.165	.094	.477	.463	—
RNA indicator	1.40	—	—	—	—	1.49	1.03	1.33	—
Precision (CVP %)	7.57	.82	—	—	—	7.03	13.1	12.4	—

CVD = coefficient of variation, calculated from the mean (M) and the standard deviation (S) of the volume distribution curve of each cell population: $\text{CVD} = \frac{S \times 100}{M}$ (%)

RDC = relative intracellular dye concentration (DIOC6 + AO) in arbitrary units (see results)

RNA indicator = mean red/green fluorescence ratio of the DIOC6/AO stain for each cell population

CVP = coefficient of variation of five determinations of separately diluted and stained samples of the same blood sample

cytes. In case of significant changes of the RDC of a cell population, separate determination with aliquot samples stained with AO or DiOC6 alone indicate which cell parameter is altered. The RDC in reticulocytes is highest when they leave the bone marrow and gradually decreases thereafter until the cells fuse with the erythrocyte cluster. The maturation can be more closely followed by defining fixed areas in the histogram (Fig. 1). The number of mature (RI), intermediate (RII) and immature (RIII) reticulocytes in these areas are calculated and displayed as a reticulocyte maturation profile (Fig. 3). The reticulocyte maturation profile of healthy adults is different e.g. from the profile of a patient during stimulated erythropoiesis after a blood loss (Fig. 3). Fig. 3 can be generated automatically during data evaluation without additional time requirements. The RDC decreases significantly from RIII to RI (Table 1) which represents the physiological maturation process of the reticulocytes. The RDC of all reticulocytes together (RI + RII + RIII) is an indicator of the mean maturation state of the whole reticulocyte population (Table 1).

The lower limit of the RI compartment in Fig. 1 constitutes an arbitrary separation between reticulocytes and erythrocytes. The position of this erythrocyte/reticulocyte threshold line was established in the following way: Aliquots of 10 blood samples were stained with brillant-cresyl-blue and the reticulocytes were microscopically counted in smears. Other aliquots of the same blood samples were stained with DiOC6/AO and measured under standardized conditions in the flow cytometer. The position of the erythrocyte/reticulocyte threshold was empirically determined by fulfilling the condition that the mean manual and the mean flow cytometric reticulocyte count calculated from the 10 samples coincided. During this empirical fitting process the threshold line was set for each fitting step at the same position for all ten samples to exclude subjective judgment. Once the position of the threshold line was determined (13 relative fluorescence units (RFU); Fig. 1), its position was expressed as fraction of the mean

fluorescence of the standardizing particles (390 RFU). The line is automatically positioned under the present staining conditions by the computer to the 3.0% level (13 : 390×100) of the mean particle fluorescence. This standardization value is valid on a day to day basis as long as the same staining conditions and particles are used for the measurements.

The AO stain and DiOC6 membrane potential stain are usually more sensitive for the detection of reticulocytes than the manual brillant-cresyl-blue reference method because the fluorescent AO and DiOC6 dyes pick up the uniform fluorescence of non particulate cellular RNA and also the higher transmembrane potential of mature reticulocytes and young erythrocytes. The erythrocyte/reticulocyte threshold is set to 13 RFU (Fig. 1) by the computer to match the manual count. The threshold would be set to 8 RFU by visual criteria. The reticulocyte count is then 2.13% instead of 1.35%.

Provided a flow cytometer capable of measuring simultaneously the cell volume and two fluorescences of each cell is available, an additional feature of the blood cell staining assay can be evaluated. The red AO fluorescence of each cell is separable from the green AO and DiOC6 fluorescences by an additional dichroic mirror (530 nm). The green fluorescence is measured between 500 and 530 nm by the first photomultiplier, and the red fluorescence between 550 and 700 nm by the second photomultiplier. The red stain of AO in lymphocytes, granulocytes and reticulocytes indicates mainly intracellular RNA and, therefore, reflects the functional activity of the cells with regard to protein synthesis or nucleic acid metabolism. Thrombocytes, reticulocytes and granulocytes usually have a significant amount of intracellular red fluorescence while lymphocytes are practically devoid of red fluorescence. It is not clear, whether the red stain of thrombocytes is a prevailing RNA stain. The increase of the ratio red/green fluorescence of the AO/DiOC6 stain of each cell reflects the red shift of the AO spectrum in presence of RNA. The DiOC6 spectrum is not involved in the spectral shift because the red/green fluorescence ratios of all cell clusters are closely similar if the cells are stained with DiOC6 alone. Quantitative values of the ratio shift of AO are given in Table 1. The values were calculated from a three parameter histogram (cell volume, red and green fluorescence of each cell) measured from the same assay of stained cells used for the measurement of the histogram of Fig. 1).

Discussion

The importance of blood cell counts for hematology is generally accepted and many different methods, both by manual or electronic counting have been developed in the past. The main intention for the development of this assay was to combine blood cell counting with molecular, biochemical analysis on a single cell level. Particular attention was paid to functional parameters because they should be sensitive indicators for the metabolic state of the blood cells in circulation. The measurement of the functional state of blood cells seems of interest, e.g., for thrombocyte dependent coagulation disorders, or in the case of cytostatic drug treatment to recognize imminent cytopenias prior to the decrease of cell counts. The stain also permits the quick evaluation of the functional state of the erythropoietic system through the maturation profile of the reticulocytes. It seems also possible to use functional blood cell stains for early biological radiation dosimetry (2 to 24 h) which may be of interest for the biological

surveillance of radiotherapy or for the diagnosis of accidental radiation insults in general (Valet, in preparation). The biochemical measurements are not restricted to transmembrane potential and RNA/DNA measurements. Esterase activity, intracellular pH, SH-groups, cellular protein and cellular lipids have also been determined in peripheral blood cells with other dyes in a similar way (Valet, in preparation).

Three advantages of the present blood cell stain seem of major interest for routine application. These are: speed, possibility for automation and recognition of the functional state of the blood cells.

The fast availability of the results is assured by a short time of staining (5 min), measurement (1 to 2 min) and evaluation (approximately 5 s in a microprocessor). Sample preparation is also fast because it only consists of a one step dilution and the single addition of a combined dye and standardizing particle cocktail. Measurement time can be probably shortened by using flow-cytometer chambers which permit higher flow rates around 5000 cells/s at a low degree of cell coincidence, instead of 1000 to 2000 cells/s with the present chamber.

The possibility for automation of sample preparation, measurement and evaluation is of importance for clinical applications. The advantage of the DiOC6/AO blood cell stain, due to its simplicity, is that its implementation in future multipurpose automated flow cytometers is possible. Such instruments will be able to measure a variety of cell samples of different origin, stained with different biochemically well defined molecular stains, similarly as photometers are capable of measuring different assays, e.g. of serum, in a clinical chemistry laboratory. The application of molecular stains (e.g. fluorescent antibodies [6], DNA [7], lectin receptor [8], protein [9], intracellular enzymes [10, 11, 12], pH [12] is not restricted to blood cells. Bone marrow cells, lymph node cells and also cells of many other organs obtained by smears, biopsies or surgery are measurable. Cells of tissue pieces can be brought efficiently into single cell suspension either by mechanical or enzymatic treatment.

The inclusion of the monosized particles in the dye cocktail is of great importance for the measurement. Monosized particles of diameters of greater than 2 μm have only recently become available [2]. The particle size and fluorescence for the blood cell stain are chosen such that they do not interfere with the cell clusters (Fig. 1). The particles fulfill several functions. They serve as an internal concentration standard, and furthermore as calibrators of the cell volume scale and the erythrocyte/reticulocyte threshold. In addition, the signals of volume and fluorescence of each particle and the coefficient of variation (CV(%) = 100 x standard deviation/mean value of the particle distribution curve) of the cell volume and fluorescence distribution curves of many particles can be used in microprocessor controlled [13], intelligent flow-cytometers to monitor automatically the instrument performance on-line during the measurement. A microprocessor controlled instrument could e.g. stop the measurement in case of orifice plugging for a moment, try to readjust or to deplug the orifice by flushing, and continue, if flushing was successful or definitively stop the measurement.

It is believed that the combination of blood cell counting with the determination of biochemically well defined molecular parameters of single cells is of general interest for hematology. Pathological conditions may be recognizable earlier or better by the biochemical state of the cells than by counting alone. The advantage of molecular blood cell counting methods is also that no additional time beyond the normal count-

ing time is required. At the present time only non automated multi-purpose flow-cytometers are commercially available. It is desirable to develop automated multi-purpose flow-cytometers in order to fully exploit the advantages of biochemical staining methods for routine and research work.

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