OPTICS OF LEUCOCYTES

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Abstract: Optical methods to study neutrophils, eosinophils, basophils, lymphocytes, and monocytes are reviewed. Recent applications of scanning flow cytometry to characterize lymphocytes and monocytes is presented.

Key words: morphology, erythrocyte, platelet, lymphocyte, monocyte, neutrophil, basophil, mononuclear, granulocyte

1. DIFFERENTIATION OF LEUCOCYTES FROM LIGHT SCATTERING

Ordinary flow cytometry has been applied to discriminate white blood cells. Granulocytes, monocytes, lymphocytes occupy unique positions in the two-dimensional space created by the forward- and side-scattered light intensities. De Grooth *et al*.[1] proposed measurement of the depolarization of side-scattered light to discriminate within the granulocyte subpopulation. Measurement of the depolarized side-scattering enables one to discriminate human eosinophilic granulocytes from neutrophilic granulocytes.[2] Finally they measured forward light scattering, orthogonal (side) light scattering, and the fluorescence intensities of unlysed peripheral blood cells labeled with CD45-phycoerythrin (CD# is cluster designation number) and the nucleic acid dyes LDS-751 and thiazole orange utilizing an ordinary flow

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cytometer. Erythrocytes, reticulocytes, platelets, neutrophils, eosinophils, basophils, monocytes, lymphocytes, nucleated erythrocytes, and immature nucleated cells occupy unique positions in the five-dimensional space created by the listmode storage of the five independent parameters.[3] Such a technique is implemented in cytometrical protocol, employing higher depolarized orthogonal light scatter of eosinophils.[4,5,6] Unfortunately light scattering measured in a fixed solid angle does not provide characterization of blood cells, i.e. determination of cell characteristics from light-scattering data. In order to systematically study optical properties of nucleated blood cells we must increase the quantity and improve the quality of light-scattering information read from individual cells.

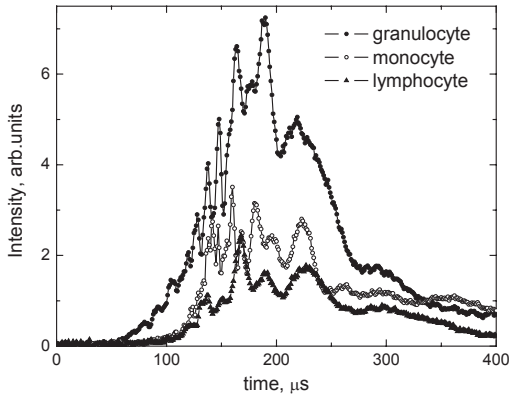

Figure 1. Examples of LSTs of three basic types of leucocytes: granulocyte, monocyte, and lymphocyte. The zero point in time scale corresponds to about 90^0, 100 μs – 55^0, 150 μs – 23^0, 225 μs – 10^0, 400 μs – 5^0.

In the technique of scanning flow cytometry, the angular scattering profile of a moving particle is measured as a function time as the regions of the scattered field are reflected by a spherical mirror to a detector. The light-scattering trace (LST) contains both the forward-scattering (FSC) and side-scattering (SSC) signals of individual particles, i.e. the temporal dependence of the scattering intensity of the moving particles. The LST can be transformed into a light-scattering profile (LSP), i.e. the scattering phase function or scattering intensity as a function of angle. This transformation and detailed description of the scanning flow cytometry technique are described elsewhere.[7,8,9,10] Examples of LSTs of leukocytes are shown in *Figure 1*. The integrals of the LST over different angular ranges are a superior alternative to the biparametric FSC/SSC plots, since they provide much more data. Three populations of leukocytes can be gated out (*Figure 2*) using the two integrals of the time-resolved LST: the integral from 350 μs to 400 μs corresponds to the FSC of ordinary cytometers and the integral

from 0 μs to 200 μs corresponds to SSC of ordinary cytometers. The LST provides an opportunity to select a larger quantity of parameters, up to the number of measuring points, for differentiation of leukocyte or others cells.

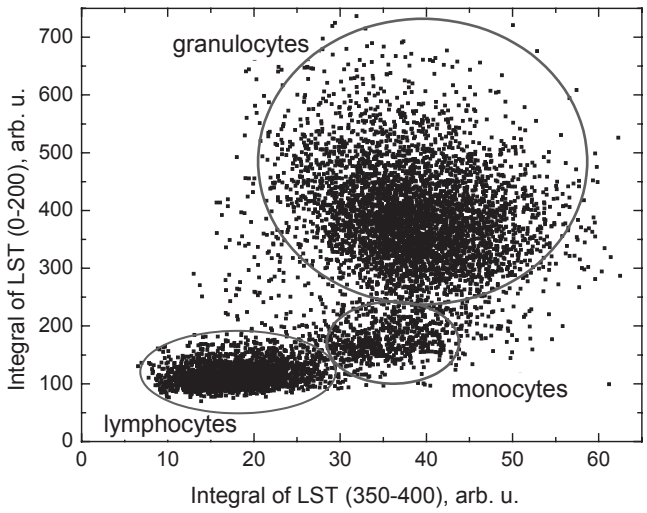

Figure 2. A leukocytes biparametric diagram of a light-scattering trace integral over 350-400 μs versus the light-scattering trace integral over 0-200 μs.

2. LEUCOCYTE SIZING

Since the Scanning Flow Cytometer (SFC) increases the amount of light-scattering data read from individual cells we are able to test the applicability of the spectral approach[11] in sizing leucocytes. The spectral approach assumes transformation of the LSP with a standard Hanning window procedure and FFT to produce a modified LSP. The typical modified LSP of leucocytes and their spectra are shown in *Figure 3*. The location of the peak P_f is used for calculating the volume-equivalent-sphere-diameter d of a cell:

$$\alpha = 189.12 \cdot P_f , \quad (1)$$

where $\alpha = m_0 \pi d/\lambda$, m_0 is the refractive index of the medium, λ is the wavelength of the incident light in vacuum.

The LSPs of individual blood cells are measured with a SFC and the fast Fourier transform (FFT) procedure was applied to the modified LSPs. The resulting spectrum is formed by a number of peaks. In order to determine a

size of the cell the location of the maximal frequency peak in the spectrum must be identified.[9] The threshold amplitude A_{th} that cuts noisy peaks from the LSP structure peaks can be estimated for each LSP with the following formulae:

$$A_{th} = 0.005M + 0.0006, \quad (2)$$

where the first term is due to ~5% noise of the laser and the second term is due to noise of the A/D converter and background. Coefficient M is the mean value of the measured signal. The typical light-scattering profiles of three types of leucocytes: granulocytes, monocytes and lymphocytes and their spectra are shown in *Figure 3*. The horizontal line in the spectrum plot corresponds to the threshold amplitude A_{th}. The location of the maximal frequency peak P_f of the spectrum is used in equation (1) for the calculation of the effective size of leucocytes. We identify leukocyte types accordingly to the integral×size map shown in *Figure 4* (a), where the integral is an integral of the LSP from 10 to 70 degrees. Each point on this map corresponds to a single leukocyte. The resulting size distributions for different types of leucocytes are shown in *Figure 4* (b). The mean size d and standard deviation s.d. are as follows: d = 8.0 μm and s.d. = 2.0 μm for lymphocytes; d = 10.8 μm and s.d. = 1.5 μm for monocytes; d = 12.4 μm and s.d. = 2.3 μm for granulocytes. The measured mean sizes of leucocytes are in agreement with literature data.[12,13]

Similarity of plots in *Figure 2* and in *Figure 4 (*a) confirms the well-known fact that forward light scattering strongly correlates with cell size. However the dots in *Figure 4* (a) demonstrate a correlation between cell size and the integral of the LSP; whereas, the analogous correlation is not observed in *Figure 2.* Assuming a correlation between the LSP integral and the size of the cellular nucleus we are able to conclude that large cells have large nuclei. This statement should be investigated with independent technique such as a light microscopy.

3. OPTICS OF MONONUCLEAR CELLS

White blood cells, which include granular and agranular, cells are an important part of the body's immune system, helping to destroy invading microorganisms. The lymphocytes and monocytes are agranular cells with very clear cytoplasm. The cytoplasm is transparent. Monocytes and lymphocytes are distinguished by having a nucleus that may be eccentric in location, and a relatively small amount of cytoplasm. The small ring of cytoplasm contains numerous ribosomes. The nucleus is round and large in comparison to the cell and it occupies most of it. In any case, some of the cytoplasm remains visible, generally in a lateral position.

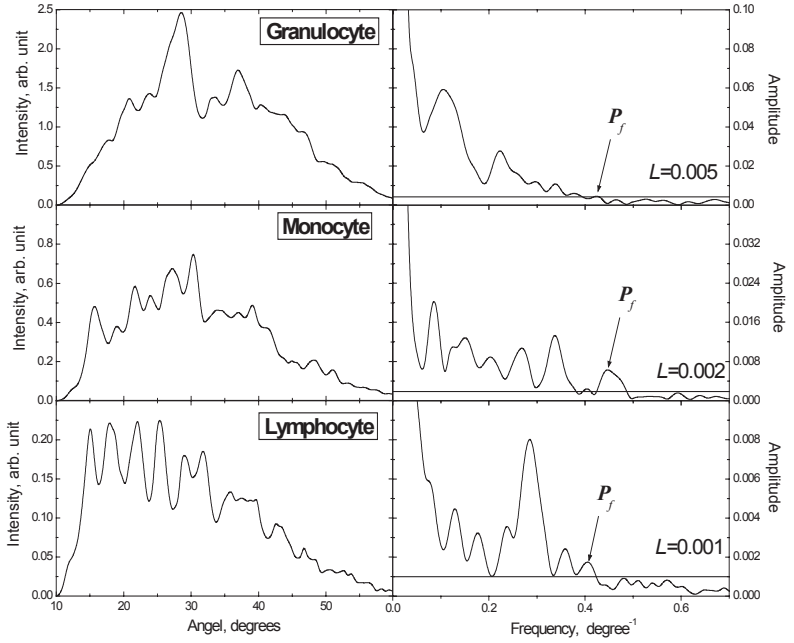

Figure 3. Typical experimental light-scattering profiles (left) and their spectra (right) for a single granulocyte (top), monocyte (middle) and lymphocyte (bottom)

In this study we analyze the light scattering of the most important subpopulation of white blood cells – lymphocytes. According to the quantity of cytoplasm, lymphocytes are divided into small, medium and large. These cells play an important role in the human immune response. The T-lymphocytes act against virus infected cells and tumor cells. The B-lymphocytes produce antibodies. Formation of the lymphocyte LSPs was analyzed with the aim of development of an appropriate optical model of a lymphocyte to solve the inverse light-scattering (ILS) problem for their characterization. The light scattering of lymphocytes was simulated by means of an algorithm[14] that allows calculations of the scattering matrix of two concentric spheres with the following characteristics: d and n_c are the diameter and refractive index of the inner sphere; D and n_s are the diameter and refractive index of the outer sphere, respectively. Additionally we were able to simulate lymphocyte light scattering with a multi-layered model[15] defined by the layer diameter d_i and layer refractive index n_i where i is the number of layer. The choice of the models is based on an analysis of experimentally measured LSPs of individual lymphocytes.[10]

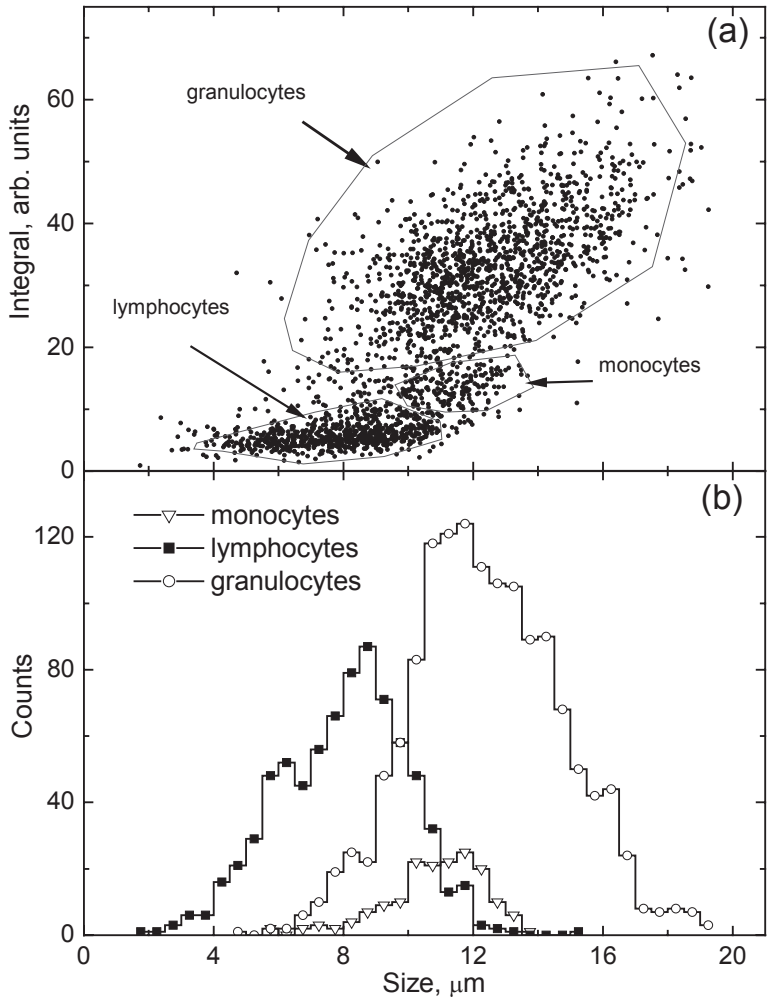

Figure 4. The (a) integral×size map and (b) the size distribution of leucocytes obtained using a SFC.

A sample that contains 5×10^5 lymphocytes was analyzed with the Scanning flow Cytometer. The spectral decomposition approach[11] was applied to the measured LPSs. The modified LSPs of two single lymphocytes with spectral decomposition are shown in *Figure 5* by points. To retrieve the lymphocyte characteristics from the measured LPS we fitted

the experimental LSPs to theoretical LSPs while varying the parameters of the two concentric spheres as follows: D and d – cell and nucleus diameters, respectively; n_c and n_s – refractive index of nucleus and cytoplasm, respectively. The results shown in *Figure 5* are as follows: (a) lymphocyte characterized by d = 6.66 μm, n_c = 1.440, D = 9.17 μm, n_s = 1.358; (c) lymphocyte characterized by d = 6.38 μm, n_c = 1.456, D = 8.74 μm, n_s = 1.355. The lymphocyte characteristics retrieved were used to calculate the modified LSP and spectral decomposition. These modified LSPs with spectral decomposition are shown in *Figure 5* by grey lines. There is substantial disagreement between theory and experiment in the figure although the location of the maximal peak in the spectrum correlates with the diameter of the inner sphere. This disagreement is probably caused by the insufficient two-layer model of a single lymphocyte.

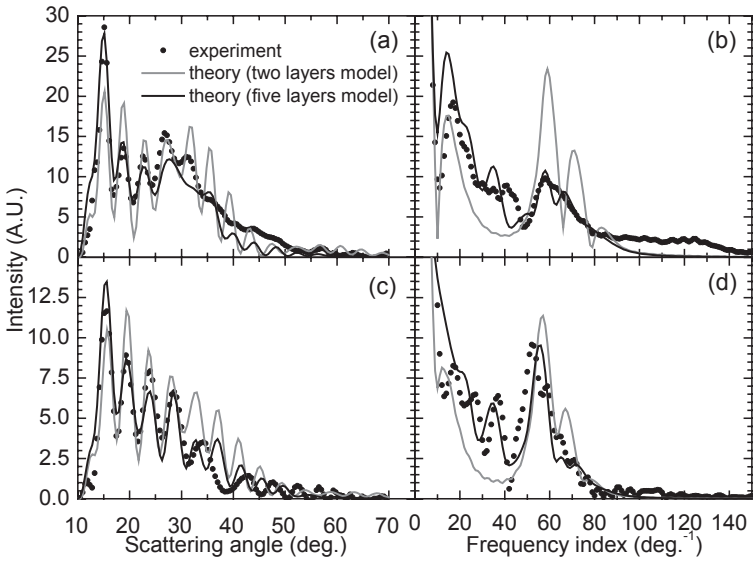

Figure 5. (a), (c) The modified light-scattering profiles and (b), (d) spectral decomposition of two single lymphocytes measured with the SFC. The experimental data is marked by points; whereas, the theoretical fits are marked by grey and black lines for two and five layers models, respectively.

In order to clarify the reason for disagreement of the theoretical and experimental results, we modeled a lymphocyte using a five-layered sphere. The experimental LSPs shown in *Figure 5* were fitted by particles formed by five layers. The resulting particles can be characterized by the following parameters: (a) lymphocyte characterized by d_1 = 1.468 μm, n_1 = 1.402, d_2 = 6.264 μm, n_2 = 1.478, d_3 = 7.895 μm, n_3 = 1.368, d_4 = 8.650 μm, n_4 =

1.360, d_5 = 9.518 μm, n_5 = 1.342; (c) lymphocyte characterized by d_1 = 1.418 μm, n_1 = 1.404, d_2 = 5.975 μm, n_2 = 1.465, d_3 = 6.606 μm, n_3 = 1.425, d_4 = 8.918 μm, n_1 = 1.366, d_5 = 9.708 μm, n_5 = 1.342. The corresponding LSPs with spectral decomposition are shown in *Figure 5* by black lines. The five-layered model evidently has given better agreement between experiment and theory. On the other hand we have found that the central part of the lymphocyte has smaller refractive index compared with the next layer.

We are assuming that the first and second layers are affected mostly by internal properties of the nucleus such as nucleolus, plaques of chromatin. The third layer is probably related with external properties defining the shape of the nucleus: indentations and over-all deviation from spherical shape. The last two layers could be related to the cytoplasm surrounding the nucleus which sometimes displays internal structures such as mitochondria and granules. We used the outer edge of the third layer as estimation for the diameter of the nucleus and the edge of the fifth layer to represent the diameter of the cell. The refractive index of the cytoplasm is considered to be equal the refractive index of the fourth layer. The refractive index of the nucleus was estimated as the volumetric average of refractive indices of the first, the second and the third layer.[16,17] The theoretical study of scattering of multi-layered spheres and experimental measurement of LSPs of individual lymphocytes has allowed us to conclude that a lymphocyte could not be modelled by two concentric spheres. The multi-layered sphere or eccentric spheres models must be applied to simulate light scattering of individual lymphocytes.

4. OPTICS OF GRANULOCYTES

Light scattering from a suspension of granulocytes was employed in dynamic studies. Light scattering intensity was used as a measure of neutrophil aggregation[18] and shape change.[19] Neutrophil degranulation was shown to correlate with the orthogonal light-scattering intensity[20] and granulocyte aggregation with light extinction.[21]

Flow cytometrical studies of neutrophil biology were reviewed by Carulli.[22] Granulocytes can be discriminated from other leukocytes in flow cytometers by their higher forward and side scattering (except basophils that are found in the light-scattering region of lymphocytes).[23] To perform further discrimination of granulocytes into subclasses, additional measured parameters are needed. One characterization method[23] employs measuring the CD45 antigen presence on the cell surface using labeled monoclonal antibodies. Eosinophils, lymphocytes, and monocytes express higher densities of CD45 than neutrophils and basophils.

Another parameter of interest is autofluorescence. Its high values for eosinophils can be used to discriminate them from all other leucocytes.[4,24] However, this parameter is not reliable, since it can be altered by chemical treatment of the cells or when using fluorescent dyes. It was combined with measurements of CD16 (specific for neutrophils) or CD49d (specific for eosinophils) to discriminate eosinophils from neutrophils.[25]

Eosinophils express several CD2 subfamily receptors on their surface: NTB-A, CD224 (2B4), CD84, CD58, and CD48. Neither basophils nor neutrophils express NTB-A. Basophils also express CD224 but not neutrophils. CD84, CD58, and CD48 are expressed by all granulocytes. Therefore, NTB-A presence can be used to identify eosinophils and CD224 absence – neutrophils.[26]

One of the first reliable methods to evaluate basophils in flow cytometry was based both on CD45 expression (low density) and on the presence of IgE on the cell surface, which can be combined with detection of low orthogonal light scattering compared to other granulocytes.[27] Improvement of this method based on the expression of CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells) / DP_2 (second receptor of prostaglandin D_2), which is also expressed by Th2 cells and eosinophils, was proposed.[28] CRTH2 detection is combined with CD3 negative (exclude Th2) and low side-scattering (exclude eosinophils) to identify basophils. The angular dependence of the light-scattering intensity was measured for granulocytes and lymphocytes. Orientation effects on the intensity of side scattering in different experimental setups were discussed.[29]

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