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A study of light scattering of mononuclear blood cells with scanning flow cytometry

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Abstract

This study describes the measurement of light scattering of human mononuclear blood cells, the development of an appropriate optical model for those cells, and solution of the inverse light-scattering problem. The angular dependency of light-scattering intensity of mononuclear blood cells was experimentally measured by means of scanning flow cytometry. A sphere consisting of several concentric homogeneous layers with different refractive indices was tested as an optical model for mononuclear blood cells. A five-layer model has given the best agreement between experimental and theoretical light-scattering profiles. The inverse light-scattering problem was solved for a five-layer model with an optimization procedure that allows one to retrieve cell parameters: cell size relates to the outer diameter of the fifth layer; size of the nucleus relates to the outer diameter of the third layer. Mean values of cell size, nuclear size, refractive indices of nucleus and cellular cytoplasm were determined for blood monocytes and lymphocytes.
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1. Introduction

Modern commercial cell counters have reached the limit of their analytical potential in cell identification (unless immunophenotyping procedures are implemented) and have a weak performance in physical cell characterization, i.e. determination of physical characteristics of cells. Blood platelet volume, red blood cell volume and hemoglobin concentration form the list of available physical characteristics measured with automatic hematology analyzers [1,2]. Leukocyte size, size of nucleus of mononuclear leukocytes, density of cytoplasm and nucleus are still out of the measurement scope of routine hematological analysis.

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A complex angular light-scattering pattern is formed from the interaction of a cell and a laser beam. The pattern is rather sensitive to cell morphology allowing determination of individual cell characteristics from a solution of the inverse light-scattering problem [3]. Unfortunately the inverse light-scattering problem was solved only for a limited number of shapes and structures of individual particles: homogeneous sphere, prolate spheroid in fixed orientation, two-layered sphere (see [3] and reference therein), biconcave discs [4]. Mathematical optimization is a common approach in solution of inverse problems. This approach utilizes an iteration procedure with multiple evaluations of the direct problem and can be effectively applied for characterization of a spherical particle with Mie theory as a solution of the direct light-scattering problem. Neukammer et al. [5] used a flow cytometer together with an ICCD camera to record the two-dimensional patterns of the light scattered by single lymphocytes. Experimental data were fitted with simulation of light scattering from homogeneous sphere with fixed refractive index. The refractive index was taken from typical value of cytoplasm (1.37), and mean diameter of lymphocyte estimated by the authors of this method was found to be $\sim$7.6 μm.

Mononuclear cells, lymphocytes and monocytes, form an important part of blood leukocytes [6]. Lymphocytes constitute about 20%–30% of leukocytes; $\sim\frac{1}{3}$ of lymphocytes has lifespan of 10–20 days, and remainder can live from hundreds of days to many years. Monocytes constitute from 0% to 10% of leukocytes, circulate for 20–40 days and then enter tissue where they mature as tissue macrophages, increase in size, morphology and carry out their principal functions. The nucleus of the monocyte is usually bean or U-shaped and is eccentric, may have a "lumpy" appearance. The cytoplasm is optically dense and usually contains fine granules.

Mononuclear cells of human blood (monocytes and lymphocytes) have been chosen for this study as simplest among blood leukocytes in morphology because they approximately have a spherical shape and contain only one nucleus. A multilayered sphere is proposed as a model to construct a solution of inverse light-scattering problem for mononuclear cells.

The theory of light scattering by a sphere consisting of two layers was first derived by Aden and Kerker [7]. Bohren and Huffman also described the procedure of scattering by a coated sphere and exhibited a calculation code along with expression formulas [8]. However their expressions could be applied only for a coated sphere. Bhandari [9] derived a complete set of scattering coefficients for multi-layered sphere. Further development of this theory and algorithm was carried out [10,11]. To calculate scattered field of a multilayered sphere we employed a recurrence algorithm introduced by Yang [12]. This algorithm is numerically stable and accurate within a large range of refractive indices and size parameters. The main features of this algorithm are decomposition of internal and external fields onto inward and outward waves and use of the first kind of Bessel function and the first kind of Hankel function. Sequentially final recursive expressions are similar to those for Mie theory for a homogeneous sphere.

In this work we have developed a new solution of the inverse light-scattering problem for a multilayered sphere that models mononuclear blood cells: monocytes, lymphocytes. There is the example of how analysis of angular dependency of light-scattering intensity of individual microparticles implemented in scanning flow cytometer can significantly extend opportunities for automatic analysis of morphological parameters in cell population.

2. Materials and methods

2.1. Experimental setup

The experimental part of this study was carried out by means of the scanning flow cytometer (SFC) that allows measurement of the angular dependency of light-scattering intensity (indicatrix) in the region ranging from 5° to 100° with respect to direction of incident laser beam. The design and basic principles of the SFC were described in detail elsewhere [3]. In this work we used an optical setup and data acquisition system described previously [13] with argon-ion laser source (488 nm, 15 mW).

2.2. Cell separation

Blood samples, using EDTA as anticoagulant, were taken from a healthy volunteer by venopuncture.

Red blood cells were lysed by incubating the blood sample with an NH_4Cl lysing solution at a 20-fold excess for 5–10 min. After that, one centrifuging step was performed at ~ 450 g for 5 min followed by one washing step with phosphate buffered saline (PBS). The resulting leukocyte suspension was diluted to a concentration about 10^6 cells/ml in PBS.

Lymphocytes and monocytes were distinguished by evaluating the light-scattering signal. In conventional flow cytometry lymphocytes and monocytes can be identified as separate clusters appearing in the "side scattering vs. forward scattering" (SSC × FSC) biparametric presentation map. Analogous to this, in SFC we retain absolute values of integral of indicatrix trace over angles from 5° to 15° and over the whole angular range, as substitution of "forward scattering" and "side scattering", respectively. Scatter plot of these two integral parameters were verified to be similar to display the same population percentages (Fig. 1) as the ones obtained by standard flow cytometry measurements (on a Coulter Epics XL). We used the scatter plot shown in Fig. 1 to first separate cells into lymphocytes and monocytes before each cell indicatrix was fitted by the inverse algorithm.

2.3. Computation of light scattering and data processing

A mononuclear cell was modeled as a multilayered sphere. We apply Yang's algorithm [12] to simulate light scattering of a multilayered sphere. In order to retrieve the multilayered sphere characteristics we used the fitting procedure based on Levenberg–Marquardt algorithm. The principle of this algorithm is optimization of the multidimensional mean square error $\chi^2(r_1,\ldots,r_L,n_1,\ldots,n_L)$ between theoretical and experimental light-scattering indicatrices. Here L is the number of layers, $r_1,\ldots,r_L$ are outer radii of spherical layers and $n_1,\ldots,n_L$ are refractive indices. Experimental indicatrices were preliminarily transformed by multiplying by $C\cdot\theta^3$, where C is a constant. Transformation works like a weighting procedure because an indicatrix has a vast dynamic range and the lower angles region, with significantly higher intensities, provide a greater contribution to χ^2 value. The indicatrix transformation allows us to distinguish the angular peculiarities for the fitting algorithm.

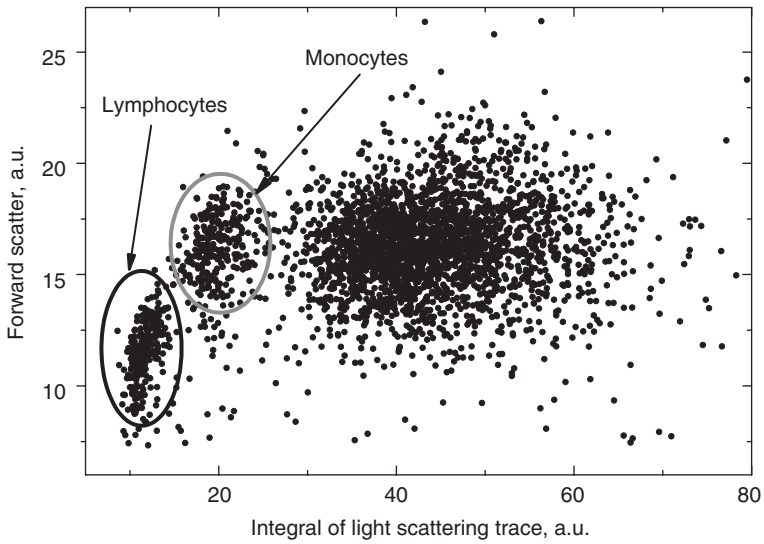

Fig. 1. Relative values of integral of indicatrix trace over angles from 5° to 15° and over the whole angular range, as substitution of the standard flow cytometric parameters: "forward scattering" and "side scattering", respectively. Resulting dot plot allows comparable identification of major leukocytic blood cell populations: i.e. lymphocytes, monocytes and polymorphonuclear cells.

The mean square error function in general depends on all $2L$ parameters of the model. In case of five-layered sphere, 10 independent parameters have to be varied. The χ^2 function has a great number of minima in 10-D space and the algorithm is sensitive to the initial coefficients, forcing us to develop an algorithm finding the global minimum of the χ^2 function. The global minimum was specified as a minimal χ^2 from a set of the local minima. The set of local minima was formed fitting experimental to theoretical indicatrix with initial coefficients which were randomly generated 50 times. In other words, we found the local minima by using different initial parameter values. The ranges of initial coefficients used in the fitting of the five-layer model of lymphocytes, the three-layer model of lymphocytes, and the five-layer model of monocytes, are shown in Tables 1, 2, and 3, respectively. Multi-dimensional space of parameters with probability density distribution to generate the initial coefficients was defined from analysis of photographs of mononuclear cells and a priori knowledge of typical cell parameters:

Outer radius of the fifth layer (r_5) was defined as uniform distribution in the size ranges reported in literature. Cell sizes are known to vary from 6.5 to 9 μm for lymphocytes and from 8.5 to 11 μm for monocytes [6,14]. The refractive index (n_5) is computed as the mean between n_4 and 1.337 ($=n$ of surrounding saline).

Width of the fourth layer ($r_4 - r_3$) is thought to be normally distributed with mean 1.2 μm and width 0.6 μm. Refractive index (n_4) is thought to be normally distributed within literature limits for cytoplasm ($= 1.3572 \pm 0.002$).

Table 1
Principles of initial parameters assignment for five-layered model of lymphocyte

Layer number	Size parameter (μm)	Type of distribution	Refractive index	Type of distribution
1	$D_1 = 0.1-(d_2 - 0.1)$	Uniform	$n_1 = 1.40-1.50$	Uniform
2	$d_3 - d_2 = 0.1-1.3$	Normal	$n_2 = 1.38-1.48$	Uniform
3	$d_3 = 0.7 \cdot d_5$	Uniform	$n_3 = \frac{n_4 + n_2}{2}$	Uniform
4	$d_5 - d_4 = 0.9-1.5$	Normal	$n_4 = 1.3570-1.3574$	Normal
5	$d_5 = 6.5-9.5$	Uniform	$N_5 = n_{\text{med}} - n_4$	Uniform

In all instances of normal distribution lower and upper limits correspond to 15% of Gaussian peak value.

Table 2
Principles of initial parameters assignment for three-layered model of lymphocyte

Layer number	Size parameter (μm)	Type of distribution	Refractive index	Type of distribution
1	$d_1 = 0.1-(d_2 - 0.1)$	Uniform	$N_1 = 1.40-1.50$	Uniform
2	$d_2 = 0.7 \cdot d_3$	Uniform	$N_2 = 1.38-1.48$	Uniform
3	$d_3 = 6.5-9.0$	Uniform	$n_3 = 1.3570-1.3574$	Normal

In all instances of normal distribution lower and upper limits correspond to 15% of Gaussian peak value.

Table 3
Principles of initial parameters assignment for five-layered model of monocyte

Layer number	Size parameter (μm)	Type of distribution	Refractive index	Type of distribution
1	$d_1 = 0.1-(d_2 - 0.1)$	Uniform	$N_1 = 1.40-1.50$	Uniform
2	$d_3 - d_2 = 0.6-1.8$	Normal	$N_2 = 1.38-1.48$	Uniform
3	$d_3 = 0.6 \cdot d_5$	Uniform	$n_3 = \frac{n_4 + n_2}{2}$	Uniform
4	$d_5 - d_4 = 0.9-1.5$	Normal	$n_4 = 1.3570-1.3574$	Normal
5	$d_5 = 8.5-11$	Uniform	$N_5 = n_{\text{med}} - n_4$	Uniform

In all instances of normal distribution lower and upper limits correspond to 15% of Gaussian peak value.

The third layer is associated with the nucleus. Little literature information is available about absolute sizes of nuclei of human monocytes and lymphocytes., However, a parameter in common use amongst cytologists to characterize cells is the nucleus to cytoplasm ratio ($N/C \equiv r_3/r_5$). N/C is a characteristic of linear dimension of nucleus divided by that of the whole cell. Analyzing photographs of mononuclear cells we derived $N/C \approx 0.7$ for lymphocytes and $N/C \approx 0.55$ for monocytes. Refractive index (n_3) is computed as average between n_4 and n_2.

The first and second layers describe the nucleus which contains chromatin consisting of DNA and protein. Biomolecules such as proteins and nucleic acids have a refractive index between 1.45 and 1.48. Values of refractive indices (n_1, n_2) are defined to be uniformly distributed between 1.4 and 1.5. The width of second layer (r_3-r_2) has a uniform distribution from 0.1 to 1.3 μm. The size of first layer r_1 is distributed uniformly within remaining limits: from 0.1 to ($r_2-0.1$ μm).

The characteristics of multilayered sphere of theoretical indicatrix that gave the global minimum of χ^2 function were assigned to the characteristics of the measured cell.

The fitting algorithm processed 1000 indicatrices of cells in approximately 3800 min on an AMD Athlon 3200 + processor (2.1 GHz). The average time to process one indicatrix of cell with 50 different initial coefficients is estimated to be 3.7 min.

3. Results and discussion

Thousand experimentally measured light-scattering indicatrices of monocytes and lymphocytes were processed with the fitting algorithm implementing the model of a multilayered sphere.

At the first stage of the study we analyzed the applicability of the three- and five-layer models to fit the experimental indicatrix of a lymphocyte. The three-layer model was based on the assumption that there are three main inhomogeneities in a cell: nucleolus, nucleus, and cytoplasm. On the other hand a native cell is not perfectly spherical making the five-layer model worth considering. The schematic lay-out of the model and cell

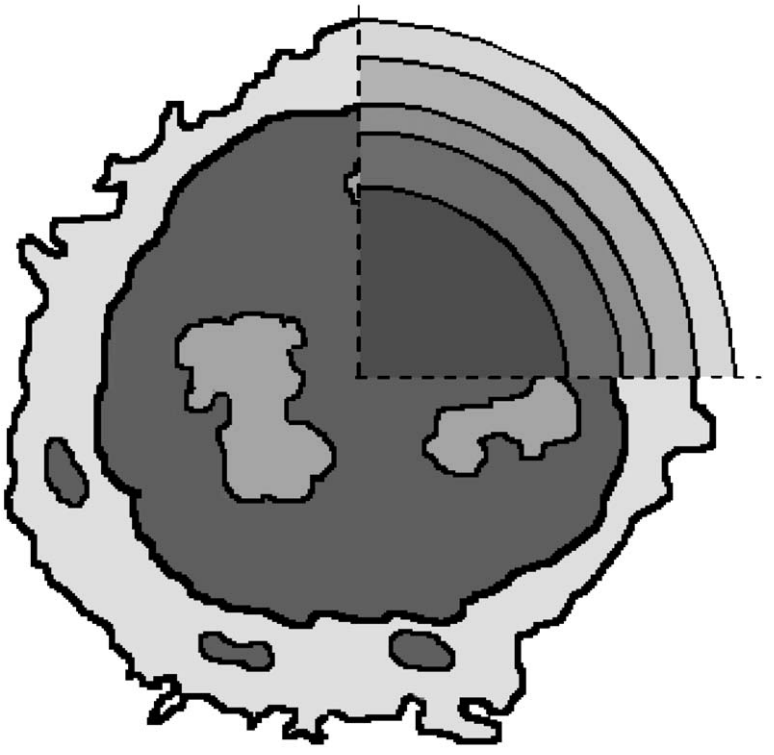

Fig. 2. Sketch of mononuclear cell and associated model of multilayered sphere.

is shown in Fig. 2. The first and second layers are mostly affected 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. The shape of the cell's periphery is sometimes far from smooth, being composed of microvilli and protuberances which are likely to affect the parameters of the fifth layer. The example of comparison of applicability of three- and five-layered models to fit the experimental non-weighted indicatrix of a lymphocyte is shown in Fig. 3. The five-layered model allows better correspondence with position and amplitude of peaks of experimental data. Numerical values of parameters of layers for given example are presented in Table 4. A less than five layers model gives significantly greater mean square difference between the theoretical and experimental indicatrices for mononuclear cells (Fig. 4). A six layer (and more) model results in errors of layer parameters exceeding their relative values for both lymphocytes and monocytes.

We used the outer edge of the third layer as an estimate for the dimension 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 to that of the fourth layer. Refractive index of nucleus was estimated as volumetric average of the first, the second and the third layer.

Averaged values of the parameters of the five-layered model computed by the proposed method for mononuclear cells within one sample are summarized in Table 5. One can note that, for all cell types computed, the width of the third layer is small with respect to the others. Here we relate the third layer to the

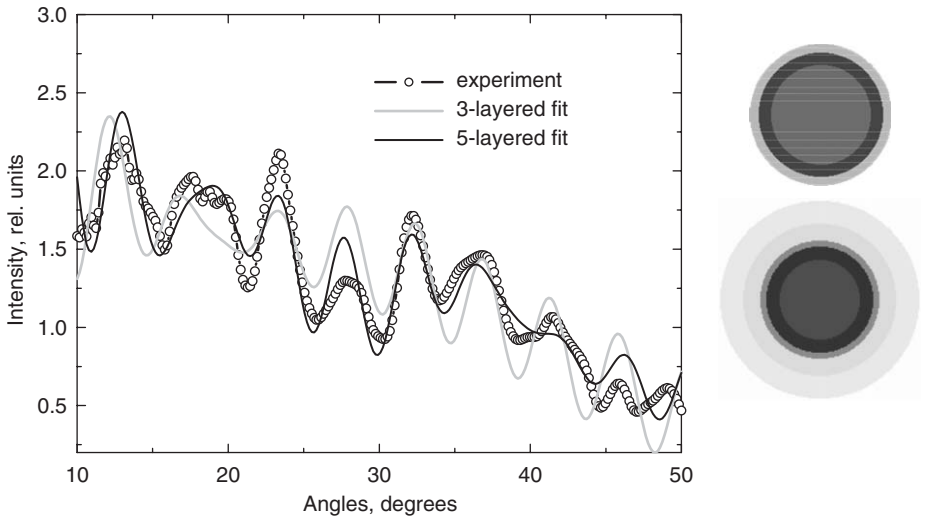

Fig. 3. Example of indicatrix signal from T lymphocyte, approximated with the three- and five-layered model. Schematic drawing of model objects produced by the best fit is shown on the right-hand side. Darker areas correspond to higher values of refractive index.

Table 4
List of parameters obtained by fitting of data from Fig. 3

Number of layer	3-layered model		5-layered model	
	Layer outer diameter (μm)	Refractive index	Layer outer diameter (μm)	Refractive index
1	3.622±0.024	1.4225±0.0007	3.113±0.014	1.4435±0.0007
2	4.431±0.022	1.4490±0.0006	4.000±0.014	1.4626±0.0005
3	5.080±0.012	1.3743±0.0013	4.456±0.007	1.3992±0.0010
4	–	–	5.623±0.029	1.3504±0.0005
5	–	–	7.158±0.027	1.3436±0.0003
Mean square error	$\chi^2 = 5.193 \times 10^{-2}$		$\chi^2 = 1.848 \times 10^{-2}$	

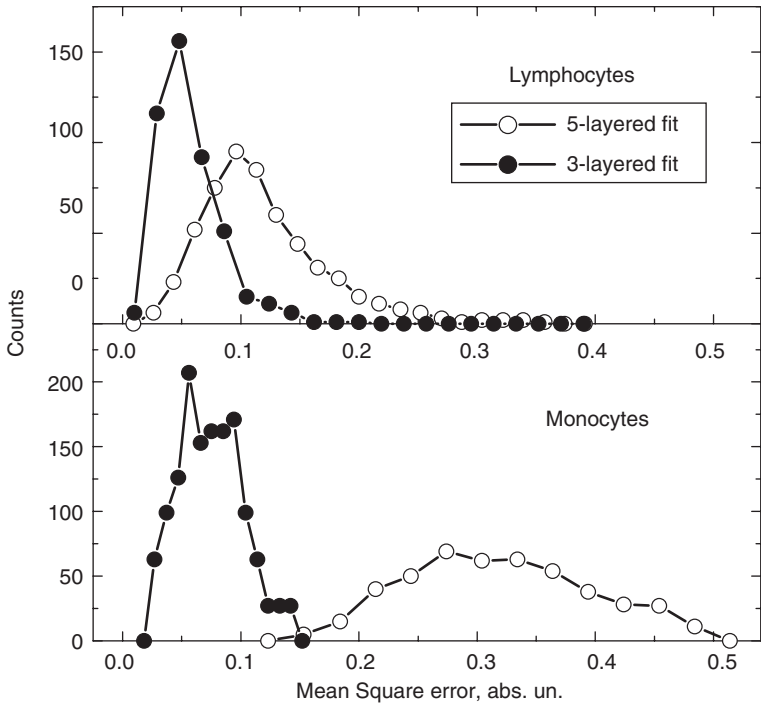

Fig. 4. Comparison of MSE distributions for data treatment by three- and five-layered models.

Table 5
Parameters of distributions for each layer of five-layered model fit for lymphocytes and monocytes

Number of layer	Layer thickness (μm)		Outer diameter (μm)		Refractive index	
	Mean	SD	Mean	SD	Mean	SD
Lymphocytes						
1	3.23	0.89	3.23	0.90	1.463	0.052
2	1.06	0.73	4.29	0.66	1.437	0.036
3	0.70	0.39	5.00	0.69	1.386	0.024
4	1.17	0.76	6.17	1.14	1.356	0.009
5	1.54	0.91	7.71	1.25	1.345	0.006
Monocytes						
1	3.65	0.89	3.65	0.89	1.444	0.056
2	0.97	0.83	4.62	0.64	1.447	0.060
3	0.58	0.41	5.20	0.59	1.394	0.043
4	4.08	1.07	9.28	1.14	1.348	0.004
5	1.01	0.66	10.3	1.18	1.344	0.010

shape of the nucleus and the observed phenomenon probably reflects the fact that a surface with high amplitude of discontinuity in the value of refractive indices greatly influences the light scattering properties of the cell.

4. Conclusion

Five-layered sphere model has been employed to simulate the light-scattering indicatrices of human mononuclear blood cells and compared to experimental indicatrices acquired by SFC. The third layer can be used to estimate the size of the cell nucleus and the fifth (outermost) one to obtain the dimension of the whole cell. The refractive index of the cytoplasm was considered to be equal to that of the fourth layer. The refractive

index of the nucleus was estimated as a volumetric average between indices of first, second and third layers. Cellular dimensions and their distribution computed in this way agree with data from literature. Thus one can expect the proposed solution of inverse light scattering problem to have some physical meaning and to reflect quantitative changes in size and morphology of mononuclear cells.

With this study we have introduced our first attempt to characterize mononuclear cells from light scattering. Certainly the new approach in characterization of blood lymphocytes and monocytes was performed in assumption that all cells are with a single nucleus and an imaginary part of refractive indices is negligible. Nevertheless our systematical study of optics of blood cells with Scanning Flow Cytometry [3] intends development of diagnostic tools for all blood cells involving most complex cells like granulocytes.

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