Determination of size parameters of isolated microparticles from data on the scattering phase function

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Scanning flow cytometer allows one to measure continuously the angular dependence of light scattering by isolated microparticles in the range of scattering angles from 10 to 70 degrees. At present, cytometers are mostly used in characterization of biological cells and bacteria in water. Their typical sizes are from fractions to tens micrometers, with the refractive indices being from 1.36 to 1.7. A simple method to determine parameters of a microparticle, based on analysis of Fourier spectrum of a modified scattering phase function, is considered. The peaks observed in Fourier spectrum are related to resonances on characteristic dimensions of particles. For the case of homogeneous spheres, this method allows one to precisely determine the particle diameter. We suggest using similar approach to characterize particles of arbitrary shapes. Results for blood cells of platelets, erythrocytes, and leukocytes are presented.

Scanning flow cytometer

The scanning flow cytometer (SFC) is an original Russian project,[1] and, in contrast to cytometers of standard configuration, it is capable of measuring continuously angular dependence of light scattering by isolated microparticles in the angular range 10 to 70° (below referred to as scattering phase function).

The operation principle of the device is as follows. The hydrofocusing head of the SFC creates two concentric flows of liquid, the inner one (diameter of 10 μm), in which the particles to be analyzed move, and the outer one (250 μm, equal to the diameter of a capillary), free of particles due to the membrane filter (diameter of the filter holes is 0.2 μm). The laser radiation is focused through an optical window into the cell. The direction of propagation of the radiation is from point 0 toward points 1 and 2.

Focusing of the laser beam into the optical cell provides for the constant irradiance of a moving particle during measurements of the scattering phase function. At any position of the particle inside the measurement zone, the spherical mirror (radius of ~ 3 mm) reflects the light scattered by the particle at a certain angle θ parallel to the flow axis. For example, angles θ_1 and θ_2 correspond to the particle position at the points 1 and 2 (Fig. 1). For a particle moving inside the detection zone of the optical cell, the scattered light is collected by the spherical mirror and then reflected parallel to the flow axis, only for scattering angles continuously varying from θ_1 to θ_2.

The parallel beams going out of the optical cell are reflected by a mirror at the angle of 45° (is not shown) and are focused by a lens to the diaphragm situated at the input window of the photomultiplier. The dependence of the voltage at the photomultiplier on time is transformed into the dependence of the intensity of scattered light by the particle on the scattering angle. The additional signal from the particle crossing the "trigger" beam (point 0 in Fig. 1) is used as a reference.

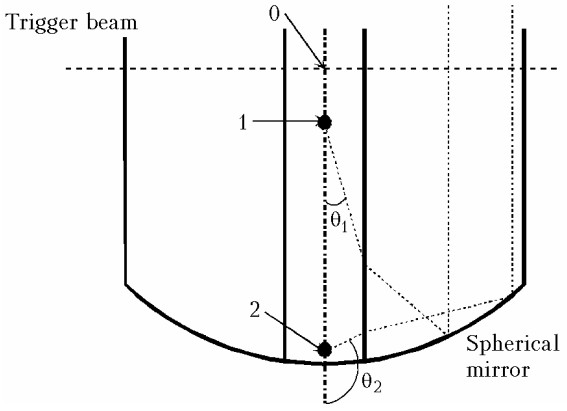

Fig. 1. Simplified diagram of operation of the optical cell. Main and trigger beams, as well as scattered beams are shown by dotted lines.

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At present, the SFC principle is used for measuring the parameters of particles in water. The wavelength of laser radiation corresponds to the visible spectral range. The range of sizes of the objects is from fractions to tens micrometers with the refractive index being from 1.36 to 1.7.

To obtain the data on the morphology of a biological cell, it is necessary to solve the inverse problem of light scattering, while calculation of even direct problem for the particle of arbitrary morphology requires, in general case, huge computer resources.

It was found in searching for simple methods of solving the inverse problem that expansion of the scattering phase function into spectrum provides for obtaining obvious data on the characteristic sizes of spherical particles.

Principle of size measurements

There are three essential aspects in the algorithm for assessing the size from the scattering phase function, which are to be clarified. Those are 1) modification of the scattering phase function for isolating peaks in its frequency spectrum; 2) relation of the positions of the peaks to the size parameters; and 3) the way of determining the "last" significant peak in the spectrum, the amplitude of which can be quite low.

Modification of the scattering phase function consists in multiplying the decoded angular dependence of light scattering by the "peak" function $F(\theta)$ defined as

$$F(\theta) = \sin^2\left(\pi\frac{\theta - 10^\circ}{70^\circ - 10^\circ}\right). \quad (1)$$

This multiplication corresponds to the filtration procedure by applying the Hunning window and removes breaks at the boundaries of the measured signal. Besides, the peak function qualitatively coincides with the transmission function of the SFC and makes optimal analysis of the obtained signals from the standpoint of the signal-to-noise ratio. All the scattering phase functions are presented below in the modified from.

The Fourier spectrum of the modified scattering phase function of a homogeneous spherical particle contains pronounced peak. In Ref. 2 equation was obtained for a homogeneous spherical particle, which relates the peak position in the spectrum of the scattering phase function and the size parameter:

$$\alpha = 189.12P_f, \quad (2)$$

where $\alpha = m_0\pi d/\lambda$, d is the particle diameter, m_0 is the refractive index of the medium, λ is the wavelength of the incident radiation; P_f is the position of the spectral peak. Equation (2) is accurate to 3% for the spherical particles with the size parameters from 8 to 100 and the refractive indices in the water medium from 1.37 to 1.7 without absorption at the wavelengths in the visible range. This range completely covers the refractive index values of particles observed in biology. The spectrum of thus obtained scattering phase function of a two-layer particle has four peaks[3] with the fourth being related to the largest particle size. In a wide range of particle sizes and refractive indices, the scattering phase function of a sphere consisting of n layers contains n^2 peaks characterizing the resonances at corresponding pairs of layers. The position of the "last" peak corresponds to the largest size of particle.[3] One can assume that similar dependence exists in the case of non-spherical particles.

In real experiment it is necessary to remove the effect of noises. The noise produced by the laser used (output power of 20 mW, wavelength of 632.8 nm) in the frequency range we are interested in is 0.5% of the total power. Then the noise of ADC becomes significant for weak signals. It can be measured in the absence of light scattering signal (it is about 0.0006 of an arbitrary unit). Then the noise level can be calculated as $L = 0.005M + 0.0006$, where M is the maximum value of the measured signal. The last significant peak can be determined as the last peak exceeding the noise level L.

Measurement of a volume (homogeneous oblate spheroids)

Platelets are the smallest blood cells. Their normal concentration is $\sim 3 \cdot 10^8$ ml^{-1}, which is lower only than that of erythrocytes, while exceeding the concentration of other cells by an order of magnitude. The shape of a resting platelet is close to an oblate spheroid. As the main role of platelets is to provide blood coagulation, they have to be activated in response to changes in the composition of the environment; they change their shape. It is for this reason that the shape of the platelets measured *in vitro* can differ depending on the way the blood sample is prepared. In this paper the ethylenediaminetetraacetic acid was used as a anticoagulant (the widespread method of preparing blood for hematological analysis, although it modifies the morphology of platelets[4,5]).

The typical scattering phase function of a platelet and its spectrum are shown in Fig. 2. The position of the peak of the maximum amplitude was used in Eq. (2) for calculating the diameter d of the sphere having the equivalent volume $V = \pi d^3/6$.

The volume distribution of platelets of the same sample was, in addition, measured by means of a Coulter MAXM hematological analyzer (Fig. 3, dotted line).

The results of comparison of two methods for measuring are shown in Fig. 3. One can see quite good agreement between two methods in the entire volume range except for small volumes.

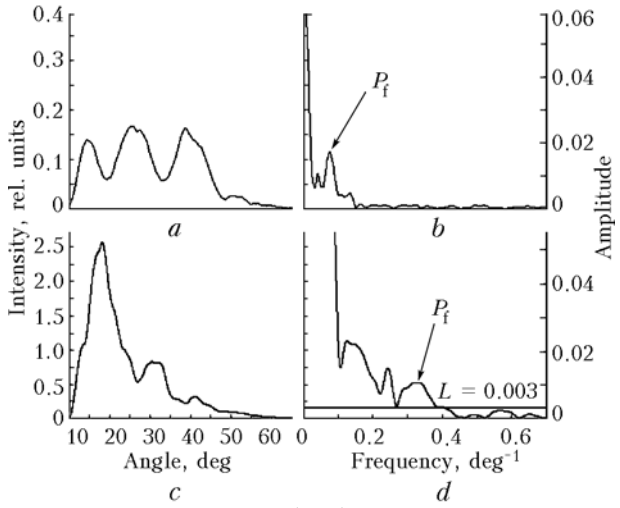

Fig. 2. Examples of the modified scattering phase functions of a platelet (*a*) and its frequency spectrum (*b*), erythrocyte (*c*, *d*). The noise level L used for determining the last significant peak in the frequency spectrum is shown by the horizontal line, P_f is the position of the peak, from which the particle size was determined.

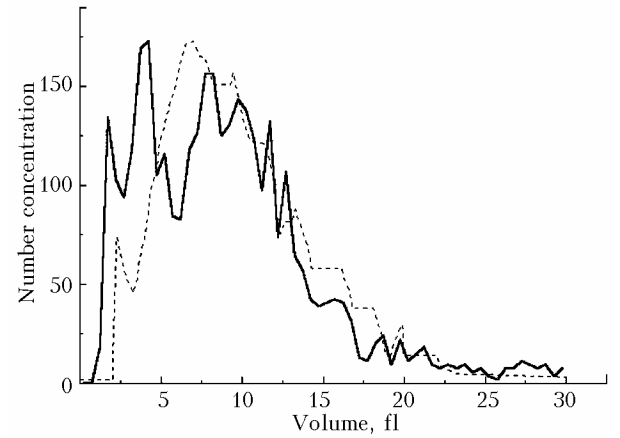

Fig. 3. Comparison of the volume distributions of platelets obtained by means of the SFC (solid line) and a Coulter MAXM analyzer (dotted line). The obtained mean values are equal to 10.3 and 9.4 fl, respectively.

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Measurement of erythrocyte size (large homogeneous biconcave disks)

The corpuscular volume of erythrocytes in human blood is about 50% (concentration of $\sim 5 \cdot 10^9$ ml^{-1}). Normal human erythrocytes have the shape of a biconcave disk. In contrast to the majority of other cells, erythrocytes of mammals have no core. As compared with platelets, which also have no core, erythrocytes are more stable to influence of external factors. The difficulties in determining the parameters of these cells are related by the fact that solving the direct problem for that large non-spherical objects needs for huge computer resources.

As was found in preliminary simulation in the approximation of discrete dipoles, the spectrum of erythrocytes can have from one to three peaks. The last peak position corresponds to the biconcave disk diameter. In simulating, the erythrocytes were considered oriented by the edge toward the incident radiation (or, the same, along the flow axis). The orientation effect is related to the peculiarities of operation of the hydrofocusing system of the device, and the estimated deviation of orientation of erythrocytes lies within the limit of a few degrees.

An example of the scattering phase function of erythrocytes and its spectrum are shown in Figs. 2*c* and *d*. Position of the last significant peak was determined taking into account the noise level L and was substituted into Eq. (2) for calculating the effective diameter of an erythrocyte d. The mean value obtained from the diameter distribution (is not shown) is 6.7 μm and the standard deviation is 1.1 μm, that is within the frameworks of the values known from literature.

Measurement of leukocyte size (cells with the shape close to spherical and complicated structure)

The leukocyte (white corpuscles) concentration is an order of magnitude lower than concentrations of erythrocytes and platelets, but these cells are the most important for diagnostics. Neutrophils (60–80% of leukocytes), lymphocytes (20–40%), and monocytes (0–10%) have the highest concentration in an organism. It is known that it is easy to distinguish among these sub-classes of leukocytes using light scattering.

All leukocytes contain a core. The lymphocyte morphology is the simplest, i.e., a large core of the shape close to spherical, and a thin layer of relatively transparent cytoplasm around it. A monocyte has more complicated, beanlike shape, the cytoplasm surrounding it is optically denser than that around a lymphocyte, and usually it contains granules. A neutrophil has the most complicated structure for optics. Its core consists of 2 to 5 large fractions of arbitrary shape and size situated quite far from each other. Cytoplasm is relatively transparent, but contains large number of microgranules.

Separation of leukocytes was carried out using the "integral versus size" map shown in Fig. 4, which presents the integral of the modified scattering phase function from 10 to 70°, and the size is determined by Eq. (2). This way is analogous to the empirical separation using the "small-angle scattering – scattering at 90°" map well-known in traditional cytometry.[6]

Position of the last significant peak (by analogy to Fig. 2*d*) was used in Eq. (2) for calculating the leukocyte size. The obtained size distributions are

shown in Fig. 4*b*. The values of the mean size and the standard deviations are the following: $d = 8.0$ μm and $\sigma = 2.0$ μm for lymphocytes, $d = 10.8$ μm and $\sigma = 1.5$ μm for monocytes, and $d = 12.4$ μm and $\sigma = 2.3$ μm for neutrophils, that agrees with the literature data.

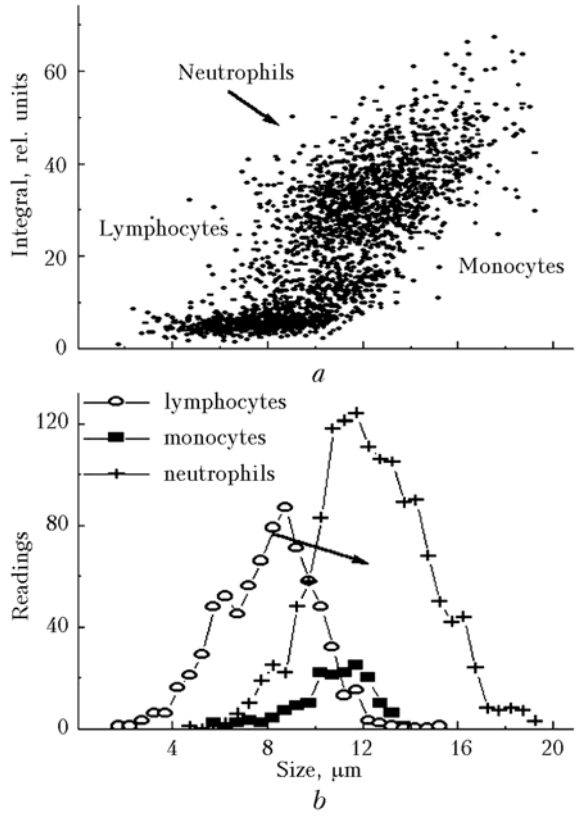

Fig. 4. The "integral versus size" map (*a*) and the leukocyte size distributions (*b*).

Thus, a simple algorithm is demonstrated for determining the size of non-homogeneous and non-spherical particles. The idea of the approach proposed is in analyzing the position of peaks of the spectral expansion of the modified scattering phase function of individual microparticles. The volumes of platelets, diameters of erythrocytes, and the size of lymphocytes, monocytes, and granulocytes have been measured using this method.

Conclusion

The practical result of this paper is the development of the algorithms for analysis of populations of the human blood cells for hematological analyzer based on the scanning flow cytometer, the first prototype of which is planned to be produced during a year. Although the presented formulas for determining the size of platelets and erythrocytes will be replaced in the nearest future by more complicated expressions based on algorithms for solving the inverse problem on the using more accurate simulation of light scattering, the proposed approach is very informative, because in some cases it provides for obtaining the size parameters of particles without accurate data on its shape. The experimentally determined parameter of the scattering phase function describing the particle size is especially important for such complicated objects as neutrophils, where even adequate statement of the direct problem on light scattering is quite complicated.

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