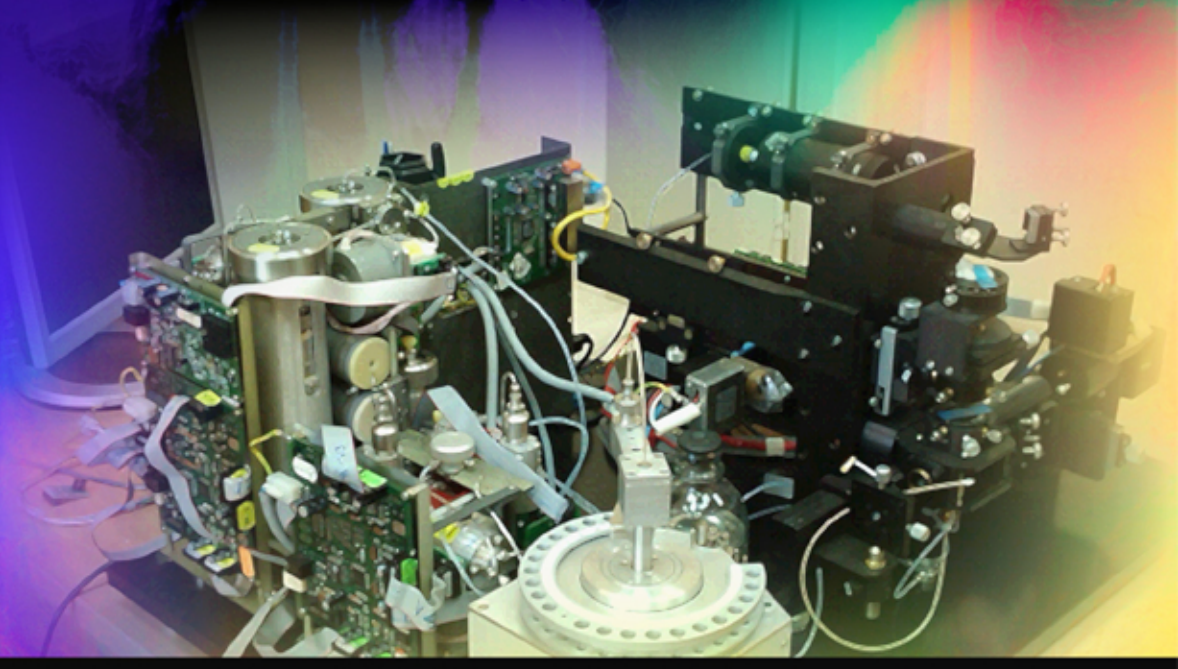
STEFANOS PAPANDREOU
EDITOR

# FLOW CYTOMETRY

## Principles, Methodology and Applications



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*Chapter 4*

# Light-Scattering Flow Cytometry: Advanced Characterization of Individual Particle Morphology

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## Abstract

Light scattering has played an important role in flow cytometry since the beginning of its technological development. Most of the significant results were demonstrated with analyses of individual particles from light scattering. However the progress in fluorescent biomarkers reduced the role of light scattering in flow-cytometer analyses substantially. Nevertheless the light scattering is still alive and we are presenting results from a new stage in the development of light-scattering analysis in flow cytometry. These new advances stem from angle-resolved methodology. With this review we show how the angle-resolved light scattering provides determination of physical characteristics of individual particles. Moreover this approach allows one to retrieve particle dimensions with a precision ranging from 10 nm to 100 nm depending on the shape complexity of a particle. This is subdiffraction precision that cannot be realized with conventional optical microscopy. Moreover the flow-cytometer analysis provides statistically proven results for characterization of cell populations because of the high rate of measurements. In order to demonstrate the advantages of angle-resolved measurement of light scattering we describe the principles of scanning flow cytometry that allows us to measure the light-scattering profiles (LSPs) of individual particles. In the development of this approach, we introduce the latest improvement that relates to simultaneous measurement of regular and polarized LSPs for one particle. The methodological goal of the angle-resolved technique is the determination of morphological characteristics of particles from a solution of the

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inverse light-scattering (ILS) problem. We introduce a few methods to solve the ILS problem utilizing the numerical algorithm in global optimization. The bisphere characteristics, sizes, and refractive indices of each sphere composing the bisphere were successfully retrieved by processing the regular and polarized LSPs. The LSPs showed a near-perfect agreement with T-matrix simulations, resulting in a 50-nm precision for sizing bispheres. We rigorously characterized individual blood microparticles, determining their sizes and refractive indices from measured LSPs. We introduce a novel approach for determination of the volume and shape of individual blood platelets modeled as oblate spheroids from angle-resolved light scattering using the flow-cytometer technique. Accurate measurement of the spheroidal aspect ratio of the platelets allowed us to discover the substantial differences between native and activated 10 μM adenosine diphosphate samples of platelets. We demonstrate a flow-cytometer method to measure the morphology of single *Escherichia coli* cells with a precision ranging from 50 nm to 250 nm and from 5 nm to 25 nm for bacteria length and diameter, respectively. High precision in the determination of cell morphology allows us to measure kinetics of intracellular processes like red-blood-cell lysis and lymphocyte apoptosis. We discuss further applications of angle-resolved light scattering using flow cytometry in the conclusion.

# Introduction

Humans encounter many different systems in their habitat that can affect their health: blood, aqua environment, aerosols, dairy products, etc. Quality control of these systems can be realized using the latest achievements in fundamental science to provide continuous improvements of life quality. A disperse system is formed by particles whose physical characteristics are different from surrounding medium. To provide advanced characterization of such disperse systems, we have to support a scientific research by modern technologies in the basic areas of science which are as follows: instrumental, theoretical and experimental. Let us consider the methods that are based on the optic phenomena to analyze particles in a homogeneous medium. These methods can be divided into the following categories: (1) methods based on the analysis of light emitted by a particle suspension; and (2) methods based on the analysis of light emitted by a single particle. Practical methods from the first category can be instrumentally realized in a simple way by shifting the analytical problems into theoretical and experimental areas. Indeed scientific publications demonstrate a complexity of theoretical considerations when light interacts with polydisperse multicomponent systems. Additional processing of experimental data requires substantial simplification of the experimental model to retrieve required particle characteristics forming the disperse system. The methods of the second category are more complex from an instrumental point of view; whereas, theoretical and experimental procedures are considerably simplified for these methods.

The problem of single-particle analysis using optical methods arises in different fields, including astronomy, remote sensing, and analysis of aerosols and emulsions [1]. The methods used in the analysis of *individual* particles, especially biological cells, can be divided into the following categories: (1) probe-field methods forming an electromagnetic field in the smallest volume, like confocal microscopy; and (2) full-field methods based on a detailed analysis of light scattered by a particle. The most powerful method that may be considered to belong to the second category is flow cytometry because the hydrodynamic focusing system

provides a means for analysis of individual particles. Flow cytometers allow for an analysis of individual particles in a flow using the following physical phenomenon: fluorescence, light scattering, and conductivity. In fact an overwhelming majority of flow cytometers utilize fluorescence and light scattering to analyze individual particles carrying by a flow. There exist two levels of scientific analysis of particles in a disperse system: identification and characterization. Identification, also known as classification, refers to attributing the particles to one of several, usually predefined, classes from experimentally measured signals. Characterization refers to the quantitative measurement of a particle by its morphological and functional characteristics, which can be retrieved from a solution of the inverse problem. Evidently, characterization requires substantial scientific research to establish a mathematical relation between experimental signals and characteristics of a particle. An overwhelming majority of flow-cytometer studies solve just identification problem in the analysis of disperse systems by counting and sorting particles based on their measured intensities of fluorescence and light scattering. In the analyses, the intensity of fluorescence depends on the amount of dye molecules that are linked to the particular molecular components specifically. This method is very useful to identify biological cells with different membrane or/and intracellular proteins. In rare instances, the fluorescence is utilized to solve the characterization problem of cell analysis with the determination of the distribution of specified membrane proteins over a cell population [2, 3]. Indeed the determination of the distributions required development of an adequate mathematical model of ligand-receptor-binding kinetics and solution of the inverse problem using fluorescence measurement and global optimization [4]. This approach allows the determination of a rate constant of the ligand-receptor binding that relates to the characterization of cell population.

In this chapter we would like to clarify the current state of the art of analysis of individual particles from light scattering with flow cytometry by focusing on the solution of the characterization problem. What do we mean by the solution of characterization problem from light scattering? In comparison to fluorescence the light scattering is much complex phenomena from instrumental, theoretical and experimental points of view. Characteristics of scattered light depend on the physical characteristics of a particle, orientation of the particle relative to direction of illumination, and on the characteristics of incident light that include its intensity, wavelength, and polarization. The light scattered in a fixed angle is characterized by an intensity and polarization assuming the same wavelength as for incident light. The dependence of scattered-light characteristics on angle forms the spatial distribution of light scattering, or the light-scattering pattern. A particle is characterized by a volume, shape, and internal structure, that is characterized through its refractive index distribution. The orientation of the particle relative to the direction and polarization of incident light also must be taken into account. So the solution of the characterization problem from light scattering means determination of particle characteristics and orientation from the measurement of the properties of the incident light and light-scattering pattern. In order to solve the characterization problem one should keep the following trajectory: light-scattering measurement --> particle identification --> optical model of the particle --> theoretical simulation of light scattering --> solution of the inverse light-scattering problem. The first and second stages are determined by an instrument; the third and fourth stages are determined by a theory; and the last stage is determined by experimental procedures, i.e. processing of experimental data. There are not so many studies published in articles where this trajectory has been proposed. Wyatt passed this way for the first time with the Differential I and II as

instruments, with the Mie theory and Rayleigh-Gans approximation as theories, and the least-square fit as an experimental procedure [5].

In actuality, the relationship between particle characteristics and light scattering are rather complex and most of them are not available in closed analytical form. Hence any description of these relations assumes a usage of significant of mathematical definitions and expressions, calculating formulas and algorithmic codes. With this chapter we try to introduce these relations between particle characteristics and light scattering in a verbal form with references to original publications where mathematics has been introduced in detail.

# Instrument

## Light-scattering Capabilities of Ordinary Flow Cytometers

An ordinary flow cytometer allows one to measure only two numbers from light scattering. These are the intensities of forward scatter (FSC) and side scatter (SSC). By plotting these values on a two-dimensional map, one can solve the identification problem successfully, for example, for lymphocytes, monocytes and granulocytes of blood. However, the relationship between cell morphology and these two values is generally so complicated that little hope is left for a detailed characterization of disperse system. However, ordinary flow cytometry with small modifications can allow one to solve the identification problem for blood granulocytes [6]. The modification assumes the measurement of the SSC signals with and without a polarizer and plotting these intensities on a two-dimensional map to count eosinophils and neutrophils separately. This instrumental achievement was proven theoretically with what is currently the most powerful light-scattering theory, the discrete dipole approximation, which allows the simulation of light scattering of a particle with an irregular shape and internal structure [7]. Unfortunately today's experimental procedures do not provide a retrieval of characteristics of granulocytes from light scattering because of the complexity of the inner structure of these cells. In addition, there is one example where the ordinary flow cytometer characterizes an individual particle from light scattering. In order to characterize red blood cells (RBCs) with the flow cytometer as an instrument, Tycko et al. measured the light scattered at two forward scattering angles [8]. The Mie theory was applied to simulate the light scattering from RBCs, varying volume and hemoglobin concentration. The Mie theory allows the simulation of light scattering from homogeneous spherical particles, but a mature RBC has a biconcave disk shape. In order to account for this discrepancy, the original biochemical treatment was applied to spherize RBCs with a constant volume. The solution of the inverse light-scattering problem was realized by the precomputed mesh database with the linear interpolation between meshes and with a calibration by particles with known volume and refractive index. This approach was modernized with the scanning flow cytometry where calibration was eliminated from the data processing procedure [9].

## Angle-resolved Light Scattering for Identification and Characterization

The main problem of ordinary flow cytometry in characterization of individual particles from light scattering is insufficient experimental data. The FSC and SSC signals allow for the determination of particle characteristics modeled by just a homogeneous sphere in fixed bounds for size and refractive index. A logical development of ordinary flow cytometers is to measure the light scattering at multiple angles or even the angle-resolved light-scattering profile (LSP) of individual particles over a wide angular range. Unfortunately, there are only a very small number of studies where multiangle light scattering has been used in the solution of identification and characterization problems in analysis of disperse systems. We completely agree with the statement by Rajwa et al., "Owing to the immense progress in fluorescent label development, multiparameter flow cytometry moved in the last decade in the direction of adding more fluorescence detectors, rather than enhancing scatter measurements" [10]. Nevertheless angle-resolved light scattering is still alive and we anticipate that it will have a significant impact on flow cytometry, especially on the morphological characterization of biological systems.

Historically, angular-resolved flow cytometry was used initially in the solution of the identification problem either with a multiangle instrument [11] or with scanning systems in orthogonal [12] and parallel [13] configurations between illumination and flow. The angle-resolved approach can be realized in one dimensional (1D) LSPs, polar scattering angle, or two dimensional (2D) LSPs, polar and azimuthal scattering angle. Obviously 2D LSP instrument is more complex in measurement of light scattering from individual particles comparing to 1D LSP instrument. As usual recording of 2D LSP requires a high-speed matrix detector with high-photon sensitivity. Nevertheless, the 2D LSP instruments were demonstrated as the next version of the scanning flow cytometer [14], diffraction imaging flow cytometer [15, 16], and interferometric two-dimensional scattering system [17]. At present the angle-resolved light scattering still has not realized the identification potential in an analysis of disperse systems passing the lag-phase in development [18].

Let us move to consider solutions of the characterization problem with flow cytometry in the analysis of individual particles forming disperse systems. The first attempt to solve this problem was demonstrated by Bartholdi et al. [19] with a custom-made flow cytometer equipped with a rather complex multiangle detecting system. They used the Mie theory to simulate light scattering from latex spheres. Processing of experimental data was performed with a point-by point comparison of simulated and measured intensities at different scattering angles. A few samples of latex spheres were successfully characterized from their sizes, fixing the refractive index of polysterene. Unfortunately the authors did not develop a mathematical procedure to estimate the precision of sizing of spheres from the characterization method.

The next stage of development of angle-resolved light scattering and solution of the characterization problem began with the introduction of the scanning-flow cytometer (SFC) [20]. We utilized the scanning principle of Loken et al. [12], but coincided the directions of flow and illumination of a particle. Instrumentally the SFC allows for the measurement of the entire angle-resolved LSP of an individual particle in the angular range from 5 to 100 degrees using one detector and spherical mirror as a scanning system. It looks very simple compared to other experimental set-ups for measuring the angle-resolved light scattering. At present we are using the second generation of the SFC fabricated by CytoNova company (Novosibirsk,

Russia, http://cyto.kinetics.nsc.ru/) as a commercial prototype (Figure 1). The current set-up of the SFC allows us to measure three fluorescence signals in different spectral regions and two light-scattering profiles, regular and polarized, of individual particles with a rate of 300 particles per second. The SFC is equipped with three lasers with wavelengths: 405 nm and 660 nm for scattering and 488 nm for fluorescence. The third generation of the SFC is coming soon.

With the instrumental problem of the measurement of angle-resolved light scattering effectively solved, our activity focused on the development of the computational algorithms for simulation of light scattering using the latest theoretical achievements. These algorithms have to simulate light scattering of a particle with an irregular shape and an internal structure. There was just one general approach in light scattering theories – discrete dipole approximation (DDA). The main problem of the DDA simulation is the requirement of a huge amount of computer memory and high computer clock rate. Fighting this problem, we developed the effective code ADDA that allows for the simulation of light scattering from rather large particles with reasonable computing times [21, 22]. At present the ADDA allows us to simulate light scattering of the most complex blood cell, like a neutrophil [23].

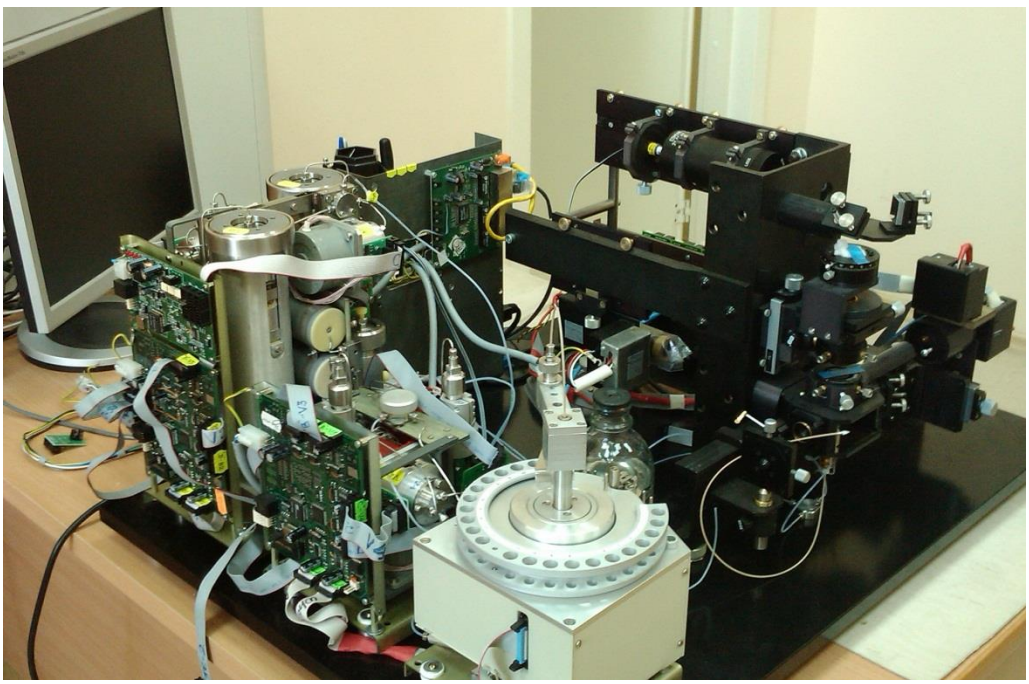

Figure 1. The commercial prototype of the scanning flow cytometer.

The final stage in the development of technology to characterize disperse systems from angle-resolved light scattering is support of this technology through effective processing of experimental data. The complex shape and inner structure of an analyzed particle requires a complex optical model of this particle with a number of physical characteristics of the particle. The homogeneous sphere characterized by size and refractive index is the simplest model that relates to polymer beads, blood microparticles, etc. Two concentric spheres which can model a mononuclear cell already have four characteristics, two diameters and two

refractive indices. A dimer formed by two spheres can be characterized by two diameters and two refractive indices but orientation of the dimer relative to direction of the incident light and polarization adds two parameters that must be retrieved from experimental data. A least-square surface formed by simulated and measured data for a particle with more than two characteristics has a substantial number of local minima. Hence, the processing of experimental data has to be done with global optimization to find the global minimum on the surface. We found that DIRECT is the most effective algorithm for global optimization [24]. We supported this algorithm with the procedure to estimate errors of the evaluated characteristics [25].

At present the solution of the characterization problem for individual particles from light scattering is supported by modern implementations in instrumental, theoretical, and experimental areas keeping in mind the SFC, the ADDA, and the DIRECT respectively. Using this approach we solved the characterization problem for a few important particles that are as follows: mononuclear cells [21], blood microparticles [26], blood platelets [27], and rod-like bacterial cells [28]. The maximal result was achieved in the solution of the characterization problem for a dimer of spheres with determination of four characteristics of the dimer and two angles of the dimer orientation [29]. Six characteristics of the inverse light-scattering (ILS) problem can only be realized with measurement of polarized light scattering.

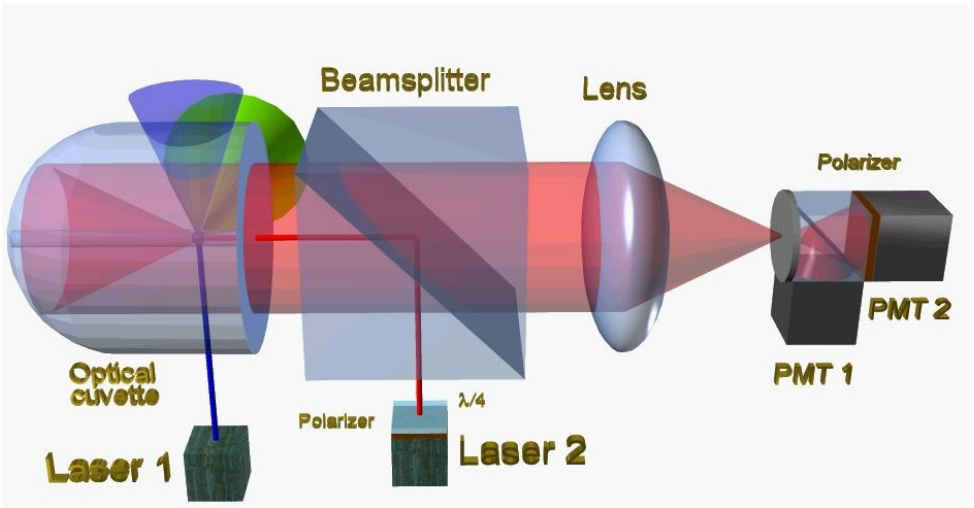


Figure 2. The schematic layout of the optical part of the scanning flow cytometer.

## Polarized Light-scattering Flow Cytometry

The current set-up of the SFC allows us to measure the regular and polarized LSPs of individual particles [25]. A detailed description of the measuring principle of the SFC can be found elsewhere [30]. The schematic layout of the SFC optics is shown in Figure 2. The left part of the scheme corresponds to an ordinary flow cytometer. The sheath and probe flow hits the capillary from the left edge of the Optical cuvette. When the particle crosses the beam from Laser 1, it emits the FSC and SSC as well as side fluorescence (shown as cones in Figure 2). In addition to the conventional orthogonal illumination, the beam from Laser 2 is

directed opposite to the direction of the flow. This beam illuminates the particle with a constant intensity when the particle is moved from the left to the right edge of the Optical cuvette in the capillary. Light (shown as a cone, a cylinder, and again a cone in Figure 2 sequentially) scattered by the particle is reflected by the spherical mirror that is to the left side of the Optical cuvette and is collected by the Lens into diaphragm of the detection unit. A spherical mirror is not a perfect focuser of light because of the aberration of the spherical surface. As a result, there is just one angle for any particle location on the capillary axis at which the scattered light will be reflected in a direction parallel to the flow and hence focused by the lens to detector unit. Schematically this function is shown in Figure 3 (left).

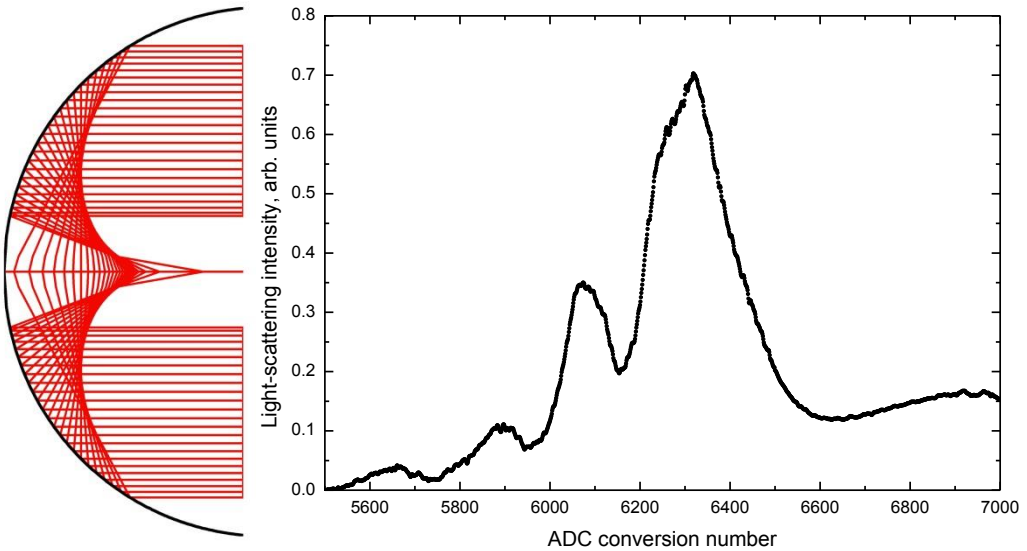


Figure 3. The SFC principle (left). The light-scattering trace of single polymer sphere (right).

Consequently when a particle is moved from the left to the right sides of the Optical cuvette (Figure 2), the detector unit measures the light scattered by this particle from 120 to 5 degrees continuously, resulting in the entire light-scattering trace shown in Figure 3 (right). Using the geometry of the optical unit of the SFC the trace can be transformed to the angle-resolved LSP [26]. The detection unit consists of two channels for measurement of regular and polarized LSPs, PMT 1 and PMT 2 with a Polarizer, respectively (Figure 2). The current from the PMTs is processed by an analog-to-digital converter (ADC). We emphasize important features of the SFC that are the integration of the scattered light over an azimuthal angle and circular polarization of the incident light of the Laser 2 formed by the Polarizer and $\lambda/4$ plate (Figure 2). From these features the regular LSP for a nonspherical particle is independent of the azimuthal angle of the particle orientation that reduces characteristics that have to be retrieved from the solution of the ILS problem by one. Finally the current setup of the SFC supports the solution of the characterization problem with the signals shown in Figure 4. Whereas an ordinary flow cytometer gives just two numbers, for example, the amplitudes of FSC and SSC, the SFC allows one to use two signatures of a particle, regular and polarized LSPs (Figure 4).

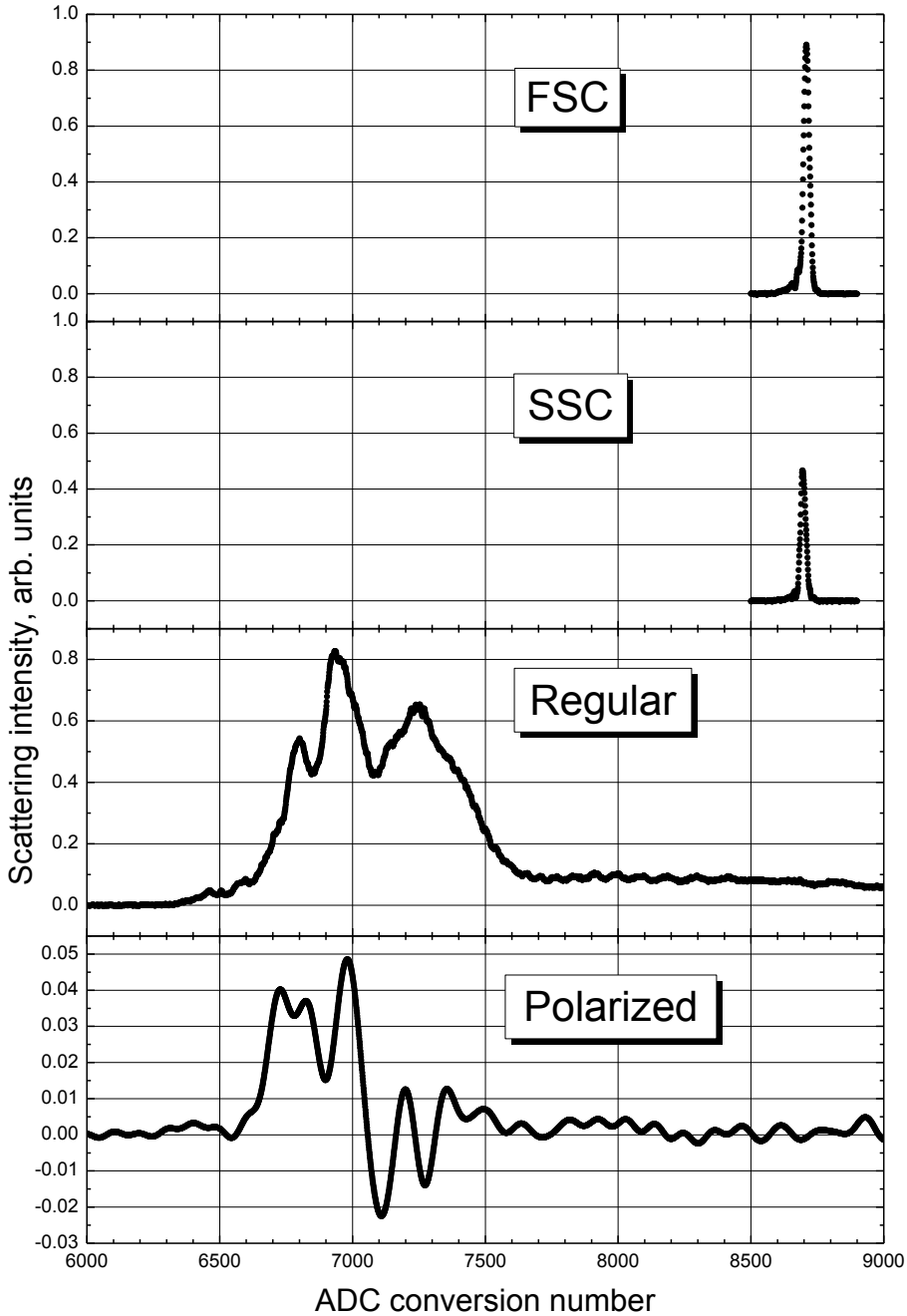


Figure 4. The measured signals of the SFC.

In order to follow the trajectory mentioned above for characterization of particles we have to develop an appropriate optical model of the particle analyzed. A more complex particle in shape and internal structure requires the development of a more complex optical model with a sufficient number of model parameters. A more complex particle model requires more independent data from light-scattering measurement. The measurement of polarized scattered light may provide up to 7 independent LSPs that will be incorporated into future

light-scattering instruments. This activity is extremely important for hematology where a solution of the ILS problem could be used for more precise characterization of blood cells. The precise characterization of blood cells assumes measurements of not only concentrations that are inherent for modern hematological analyzers, but also morphological characteristics such as volume, shape, density, nucleus size, etc.

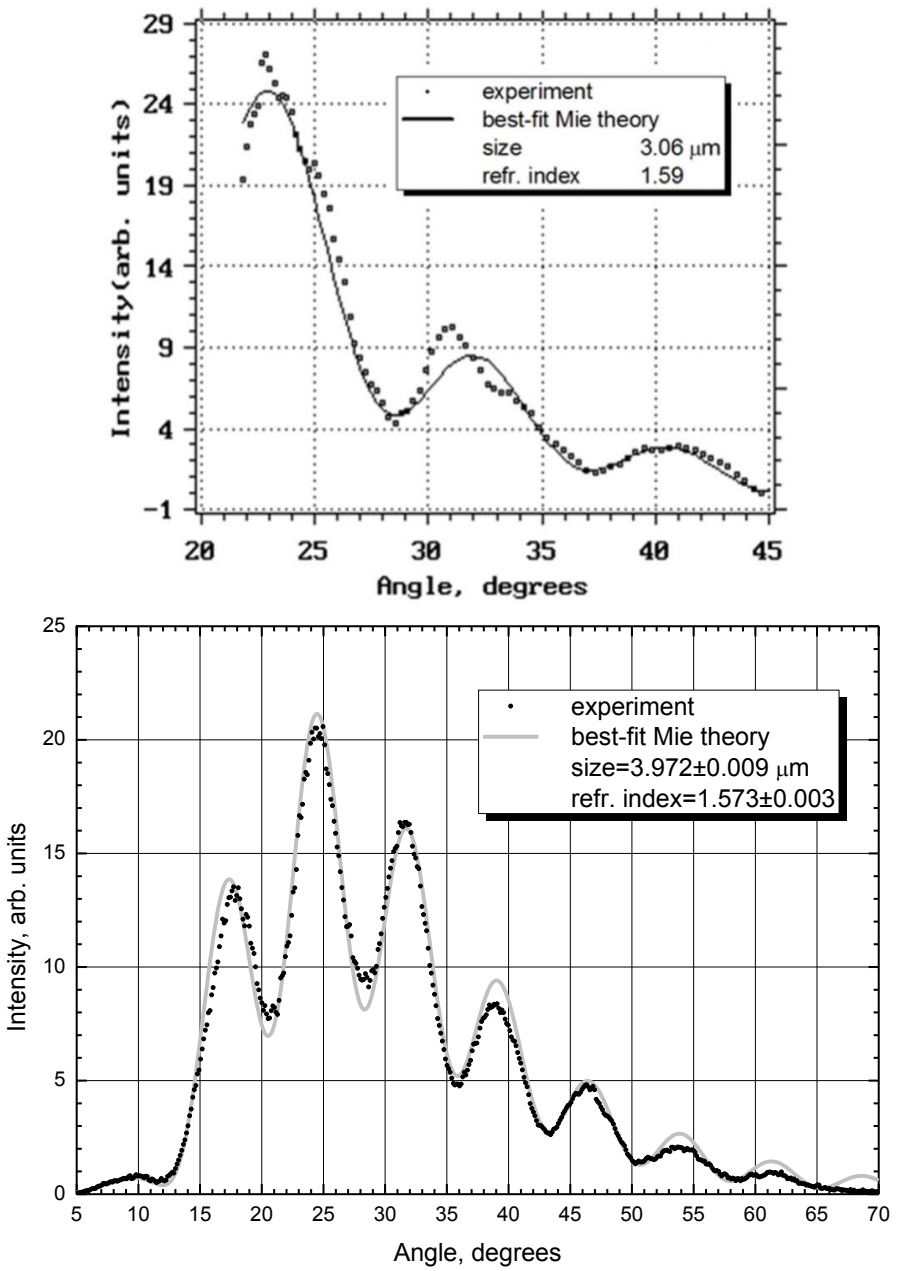


Figure 5. Agreement between experimental and theoretical LSPs measured with the SFC in 1995 (top) and in 2013 (bottom). The LSP of 2013 is shown as the weighted LSP (see text).

# Application

## Spherical Particles

### *Monomer*

Polymer beads are the most widely available spherical particles for routine analysis with light scattering and flow cytometry. Obviously, the characterization problem for spherical particles was solved by analyzing light scattering from polymer beads. The instrumental implementation of the solution was realized with ordinary flow cytometry measuring light scattering at two spatial angles and producing the two-dimensional map from the signals measured. The map was calibrated from measurement of beads with known mean size and refractive index using the Mie theory as a theoretical implementation. The processing of the experimental data was performed with an interpolation of the measured and calibrated map points.

Angle-resolved light scattering also was utilized to characterize polymer beads. Using scanning flow cytometry, the LSPs of polymer beads were introduced in first publication in 1995 [16]. The SFC demonstrated rather good agreement between the experiment and the Mie theory (Figure 5 top) but the latest experiments with polymer beads showed the near-perfect agreement that results in nanometer precision for spherical particle sizing (Figure 5 bottom). At the moment the operational angular range of the SFC optics and electronics covers the region from 5 to 70 degrees. The operation angular range is substantially expanded with the current set-up of the SFC that forces us to multiply the LSP by the weighted function [25]. The resulting weighted LSP (wLSP) can be represented with a linear scale for the light scattering intensity (Figure 5 bottom).

### *Dimer*

Characterization of a particle from light scattering assumes a solution of the ILS problem, i.e., determination of particle characteristics from light-scattering data. In general, the precision of solutions of the ILS problem depends on the amount of independent light-scattering data and the characteristics of the particle. The regular LSP can be used successfully in the solution of the ILS problem if the number of particle characteristics is increased from two to four. If the amount of particle characteristics exceeds four, the accuracy of the solution of the ILS problem drops dramatically. More independent light-scattering data must be measured to solve the ILS problem for a particle with six characteristics, like a bisphere. The solution of the ILS problem for a bisphere assumes determination of the following characteristics: radii and refractive indices of two spheres composing the dimer and two Eulerian angles. The Eulerian angles describe the orientation of a particle relative to the direction of the incident laser beam and default polarization of optical system. We solved the ILS problem for a bisphere using the SFC as an instrument, the T-matrix method [31] as a theory to simulate light scattering from a bisphere, and the global optimization with DIRECT algorithm as a procedure to process experimental data. The detailed mathematical description of analysis of dimers from regular and polarized LSPs could be found in our publication [25].

The regular and polarized wLSPs were together merged (Figure 6), and the DIRECT algorithm was applied to the joint wLSP by varying six characteristics. Finally, the solution of the ILS problem for a single bisphere allowed us to find the best-fit T-matrix LSP to

determine the four characteristics of the bisphere and the Eulerian angles. The result of the solution of the ILS problem for the bisphere is shown in Figure 6. We show the best-fit T-matrix LSP, the characteristics of the bispheres, and the Eulerian angles. The errors of determination of the bisphere characteristics and the Eulerian angles also are presented.

In our studies, we analyzed 500 dimers. The dimer spheres were sized with a mean error of 49 nm (standard deviation of 11 nm) that is extremely precise for optical methods with the wavelength from the visible region. The refractive index of dimer spheres was determined with a mean error of 0.013 (standard deviation of 0.005). The results of the measured distribution of the Eulerian angles are not unexpected: the orientation of the bisphere with angle α is equiprobable over the varying range of the angle; whereas, the orientation of bispheres with angle β is rather narrow, and the mean angle β lies close to 15 degrees. The orientation at an angle of $15^0$ corresponds to a rotation of nonspherical particles in Poiseuille flow [26] within a flow channel of the SFC.

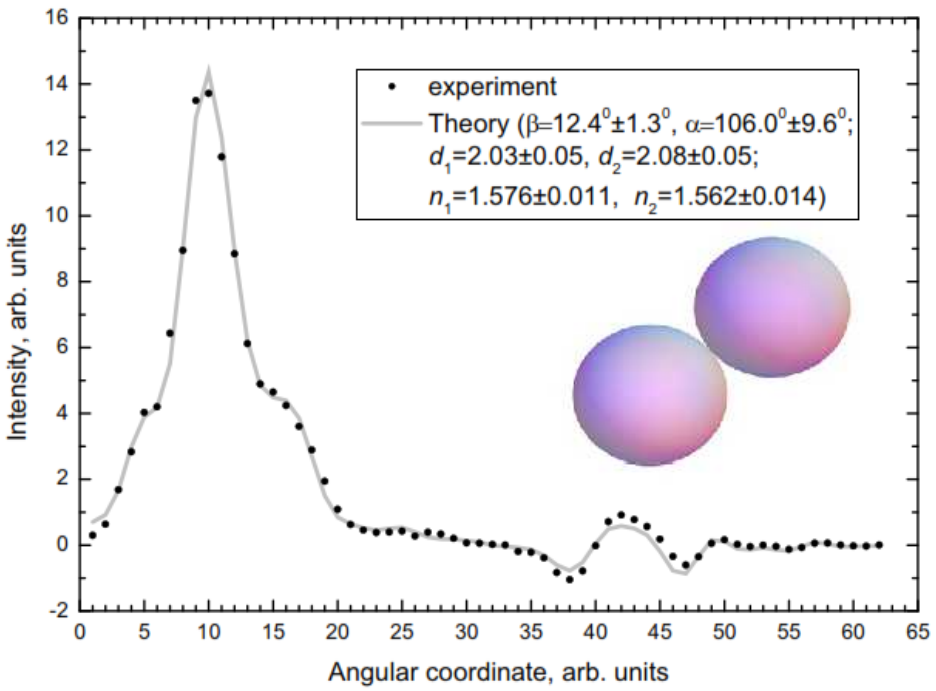


Figure 6. The solution of the inverse light-scattering problem for a bisphere. The points are the experimental merged regular and polarized wLSP. The line is the theoretical best-fit wLSP from T-matrix method.

## Rod-like Bacterial Cells

The dimer of spheres could be exactly modeled by two spheres in contact. At present this is the most complex exact optical model of particle which can be characterized from angle-resolved light scattering. There is another category of biological particles that have rather complex shape and internal structure but can be reasonably modeled be a simple physical body. For instance mature blood platelets and *E. coli* cells can be modeled by an oblate

spheroid and capped cylinder respectively. The discrepancy between a real biological particle and its optical model results in a systematic error in characterization of the particle from the solution of the ILS problem. Nevertheless, the characterization of a biological population of cells from light scattering has given very reasonable results that are comparable with a microscopic analysis of the populations [24].

We used angle-resolved light scattering to characterize individual *E. coli* cells. The SFC was used to measure the LSPs of cells, the ADDA code was used to simulate of light scattering from capped cylinder and the nearest-neighbor interpolation of a preliminarily calculated database of theoretical LSPs to process experimental data. We were not able to use the global optimization with the DIRECT algorithm because of the iterations of this method. The iteration requires multiple calculations of the direct light-scattering problem with the ADDA that require significant computations. We provide one example that shows the result of processing one wLSP of the *E. coli* cell in Figure 7. This result demonstrates better agreement between the experiment and capped cylinder in comparison to the sphere. Again the angle-resolved light scattering results in near-perfect precision for measurement of length and diameter of rod-like bacterial cells. This means that this method can be used in detailed measurement of elongation of cells during the cell cycle.

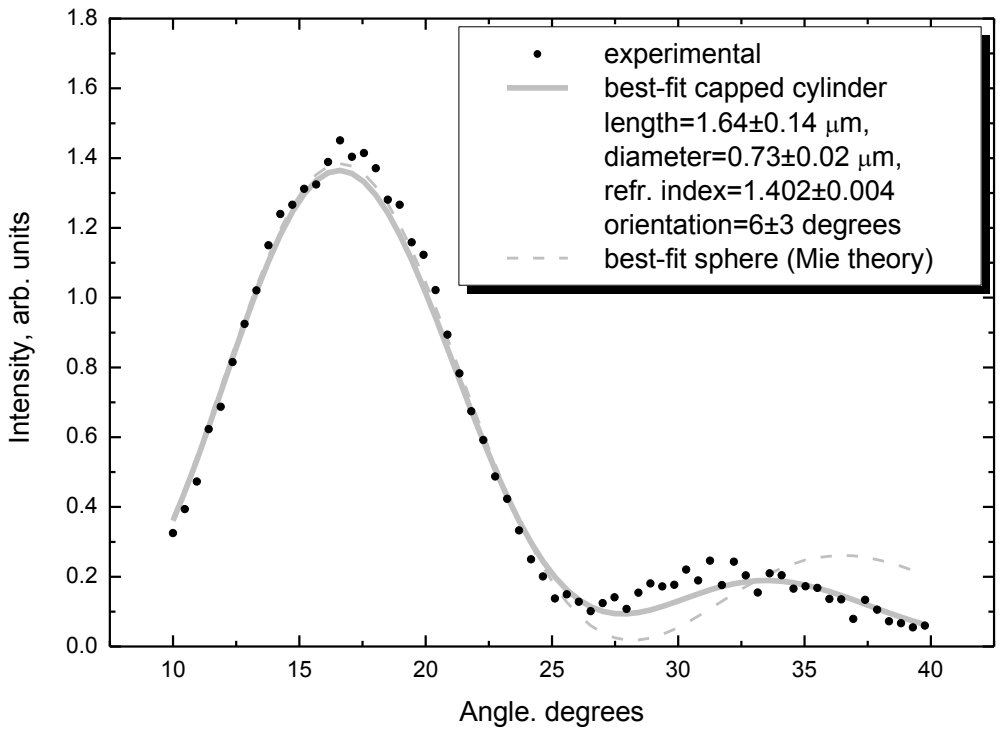


Figure 7. The experimental wLSPs of *E. coli* cell (points) and theoretical best-fit capped cylinder (solid line) and sphere (dashed line).

The method was applied to two strains of *E. coli* cells, showing 135 and 15 nm median precision in determination of length and diameter of single cells, respectively, which is very good for optical methods. We also compared population distributions over model parameters

with optical microscope measurements and obtained good agreement for both diameter and length [24].

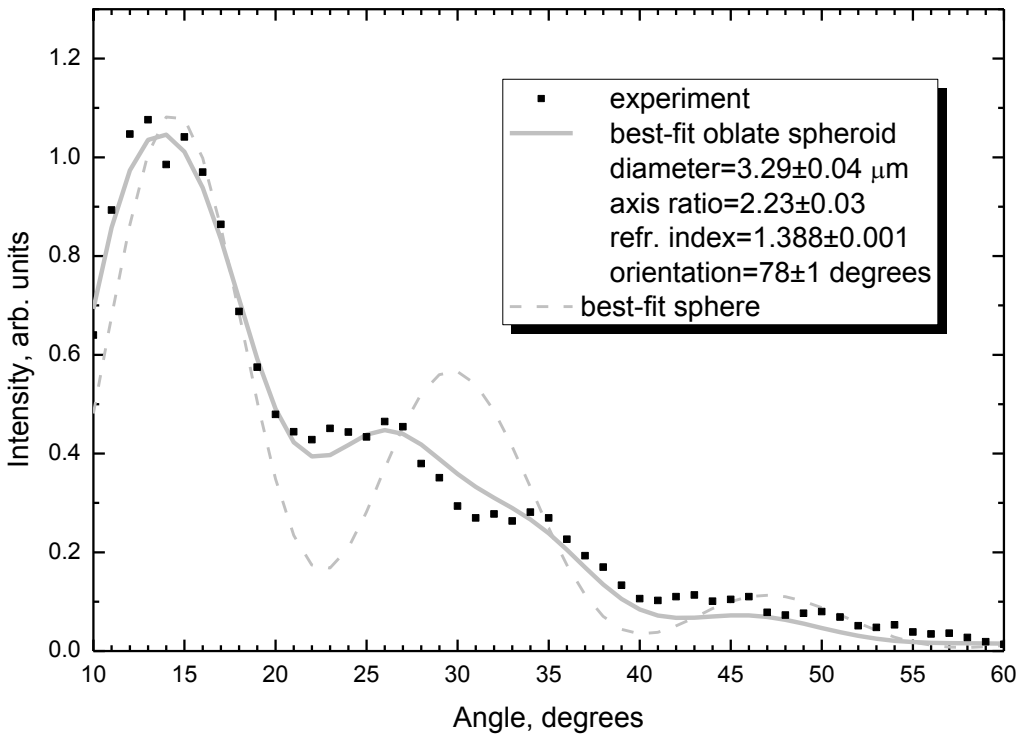


Figure 8. The experimental wLSP (points) of a blood platelet and theoretical best-fit wLSPs of oblate spheroid (line) and sphere (dashed line).

## Blood Platelets

Blood platelets have an irregular shape and internal structure but the dimensional size of platelets is close to the wavelength of visible region. Hence we are able to eliminate the small irregularities of cell membrane and internal organelles from the optical model of the cell. Using scanning electron microscopy, a platelet can be modeled by an oblate spheroid. In this way we have to remember that such optical modeling results in a systematic error in the solution of the ILS problem for a mature blood platelet. Similar to our work with *E. coli* cells, we developed the approach to characterize blood platelets by measuring the LSPs by the SFC (instrument), simulating light scattering from the DDA (theory), and performing nearest-neighbor interpolation within the LSP database (data processing) [23]. We apply this approach to blood platelets to characterize both resting and activated cells. The result of the solution of the ILS problem for one platelet is shown in Figure 8. There is rather good agreement between experiment and best-fit theoretical wLSPs; whereas, the best-fit sphere does not follow the experimental points over the angular interval measured with the SFC. Again we emphasize the precision of the determination of the diameter and the aspect ratio of the platelets. High precision is important in measurements of processes with platelets, for example platelet activation and aggregation. The high precision guarantees high sensitivity of

external processes acting on blood platelets. In particular we were able to measure a change of the mean aspect ratio for platelets after activation by adenosine diphosphate [23].

This method of characterization of blood platelets was used in the analysis of a donor's blood sample with the measured distribution over the platelet volume. The corresponding distributions over the platelet volume were in a qualitative agreement with the conventional curves measured by Coulter counters. The mean platelet volume values of 10.0 fl for mature platelets fall within the reference range of 8 to 12 fl [23].

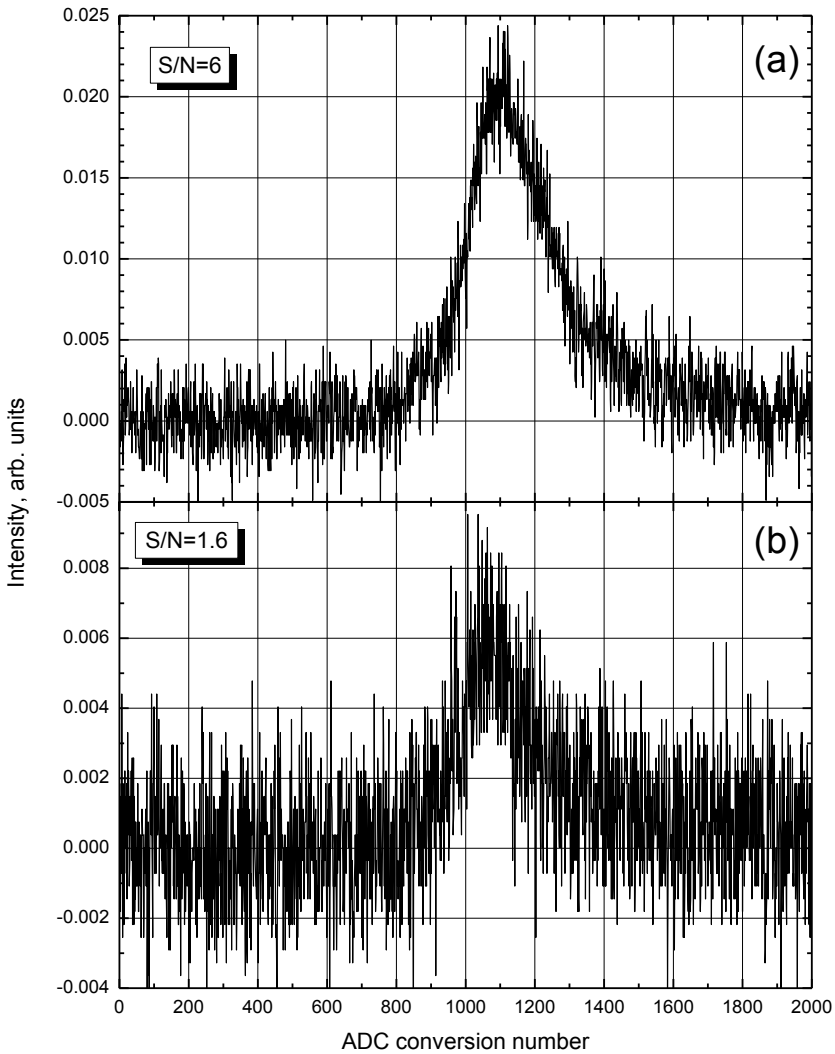


Figure 9. Light-scattering traces of blood microparticles.

## Blood Microparticles

The main problem in the measurement of angle-resolved light scattering of blood microparticles is the extremely small scattering intensity because the amplitude of the LSP

rapidly decreases with size, resulting in a natural detection limit for any particle. On the other hand, microparticles can provide an instrumental limit for individual particle characterization from light scattering. From scanning electron microscopy images, we can see that the sphere is an appropriate model for a blood microparticle. Consequently, the characterization problem can be solved with the SFC (instrument), the Mie theory (theory), and global optimization (experimental data processing).

We measured a few thousands of microparticles with the SFC. We did not expect oscillations in the LSPs because of the small size of the microparticles. In order to increase the signal-to-noise ratio (S/N), we reduced the angular resolution of the SFC by increasing the diaphragm of the detection unit (Figure 2). We note that the integration of the light scattered by a particle over azimuthal angle by the spherical mirror of the SFC helps in the minimization of the detection limit for small microparticles. We used a laser with a wavelength of 405 nm for the same also to increase the lower detection limit. There are two light-scattering traces from the detecting unit of the SFC in Figure 9. The measurement demonstrates S/N ratios of 6 and 1.6 for Figure 9 (a) and Figure 9 (b) traces respectively. These traces were transformed into the LSPs and then the microparticle characteristics were retrieved using the global optimization with DIRECT algorithm. The characterization gives the following characteristics of the micrioparticles: a size of 0.624±0.010 μm and a refractive index of 1.394±0.003 for the microparticle shown in Figure 9 (a); a size of 0.324±0.008 μm and a refractive index of 1.450±0.006 for the microparticle shown in Figure 9 (b). These experiments have shown that the current set-up of the SFC has a detection limit of 300 nm for biological particles. The detection limit for polystyrene beads can be estimated as 230 nm because of the significantly higher refractive index of 1.58 for polystyrene. This will be established in coming experiments with the SFC.

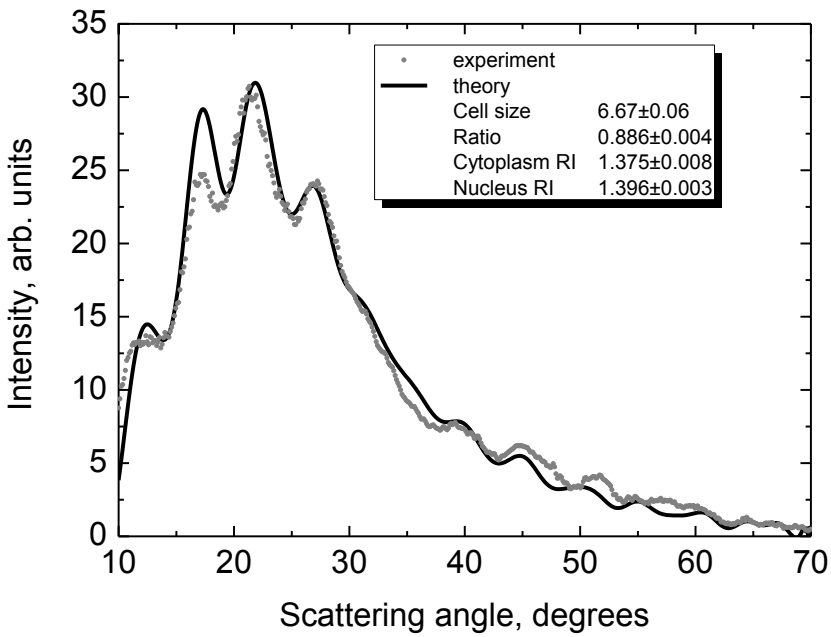


Figure 10. The experimental (points) and theoretical best-fit (line) wLSPs of lymphocyte.

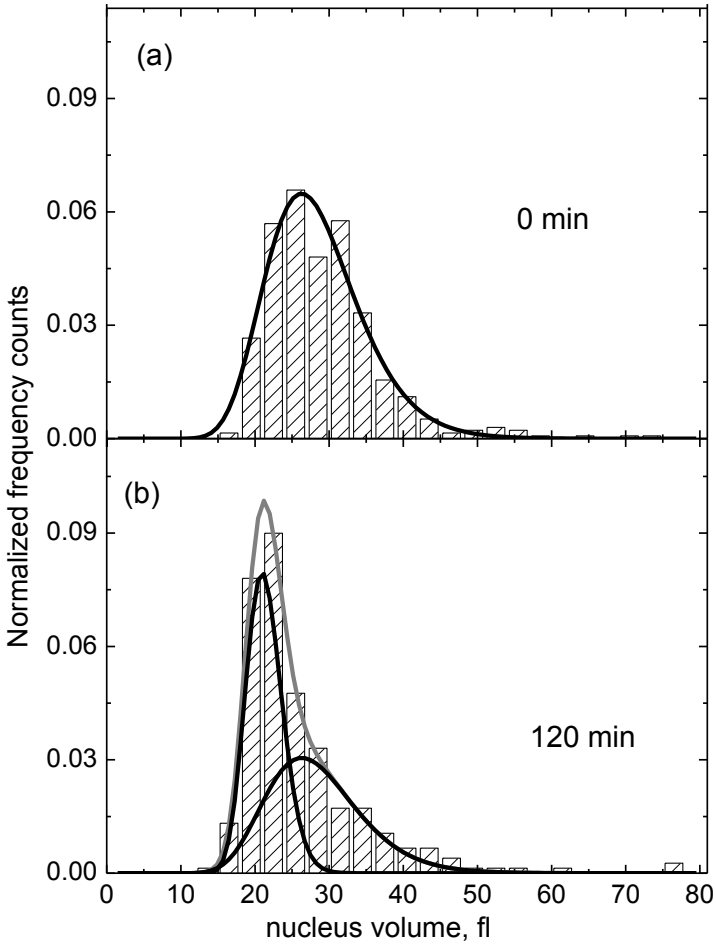


Figure 11. Distributions of lymphocyte nucleus volume (a) initial and (b) in apoptosis.

## Mononuclear Cells

At present one more category of biological particles, mononuclear cells, can be characterized from light scattering with flow cytometry. The sphere with a cover is the simplest optical model for a mononuclear cell. Obviously this model is in very rough agreement with the membrane shape and nuclear structure of a real cell, and we expect this to result in a considerable contribution of the systematic error to cellular characteristics retrieved from a solution of the ILS problem. We have shown that one primary source of systematic error arises from the inhomogeneity of the cell nucleus [21, 32]. Hence the errors of characteristics estimated from the solution of the ILS problem could be an indicator of the degree of inhomogeneity of a cell nucleus.

The characterization problem for a mononuclear cell was solved with the use of the SFC (instrument), the Mie theory (theory) and global optimization with DIRECT algorithm (processing of experimental data) [21]. The LSPs of human lymphocytes were measured with the SFC. The optical model for a mononuclear cell has four characteristics, which are as follows: the cell size, the ratio of the nucleus size to the cell size, the cytoplasm refractive

index and nucleus refractive index. An example of the characterization of one lymphocyte is shown in Figure 10. There is good agreement between the experimental points and the best-fit Mie theory curve that provides sub-diffraction-precision measurements of the cell size.

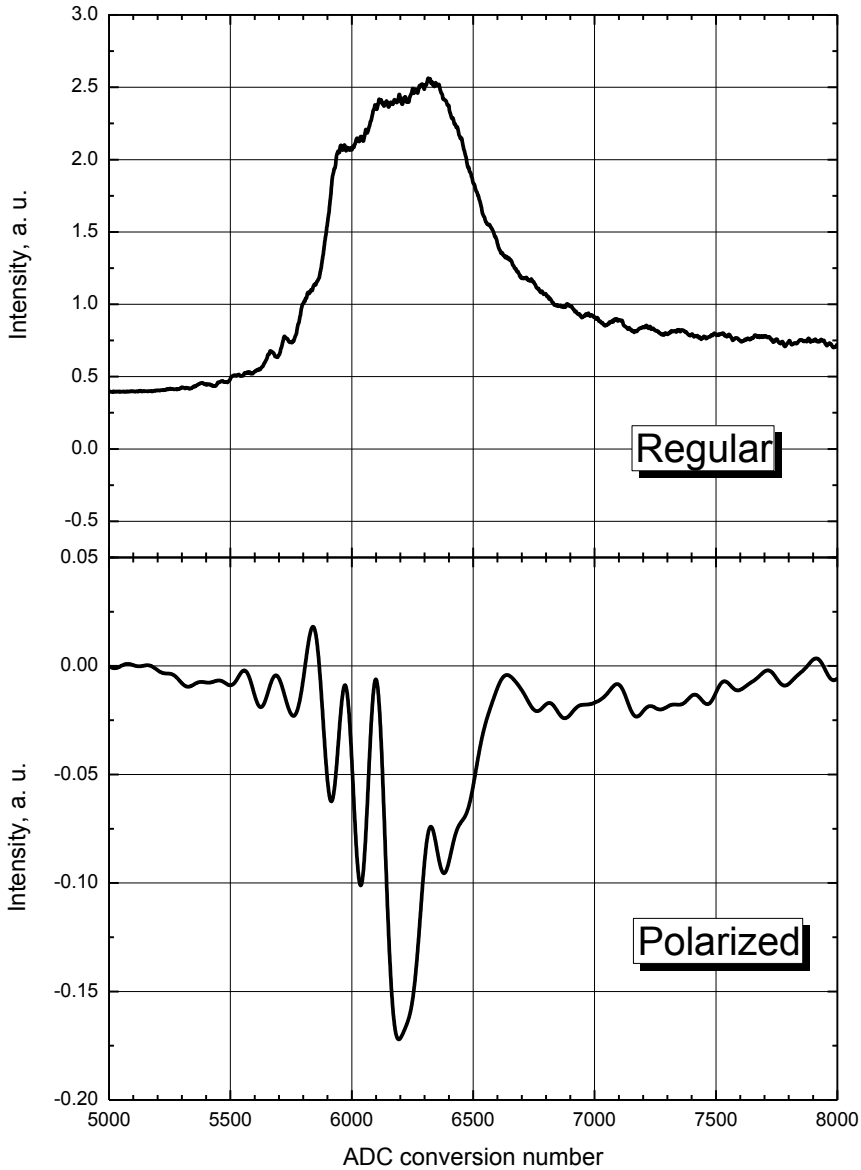


Figure 12. The regular and polarized light-scattering traces of the red blood cell.

The high precision in measurement of cell characteristics helped us to detect the differences in distributions over nucleus volume for the normal lymphocyte sample and for the sample where apoptosis was initiated. The results of this experiment are shown in Figure 11. The lognormal distribution of nucleus volume for the initial sample of lymphocytes (Figure 11 (a)) is characterized by the mean volume of 27.7±0.3 fl, the width of the distribution of 0.227±0.010 and the amplitude of 0.99±0.04. The final distribution (Figure 11

(b)) is formed by normal lymphocytes and lymphocytes with the reduced nucleus volume because of DNA fragmentation in apoptosis [33]. The lognormal distribution of apoptotic cells is characterized by the mean volume of 21.2±0.1 fl, the width of distribution of 0.115±0.004 and the amplitude of 0.48±0.01. The apoptosis was initiated for 51% of cell. This method has a potential to be used in detection of the apoptotic activity of cell population without monoclonal antibodies labeled by fluorescent molecules.

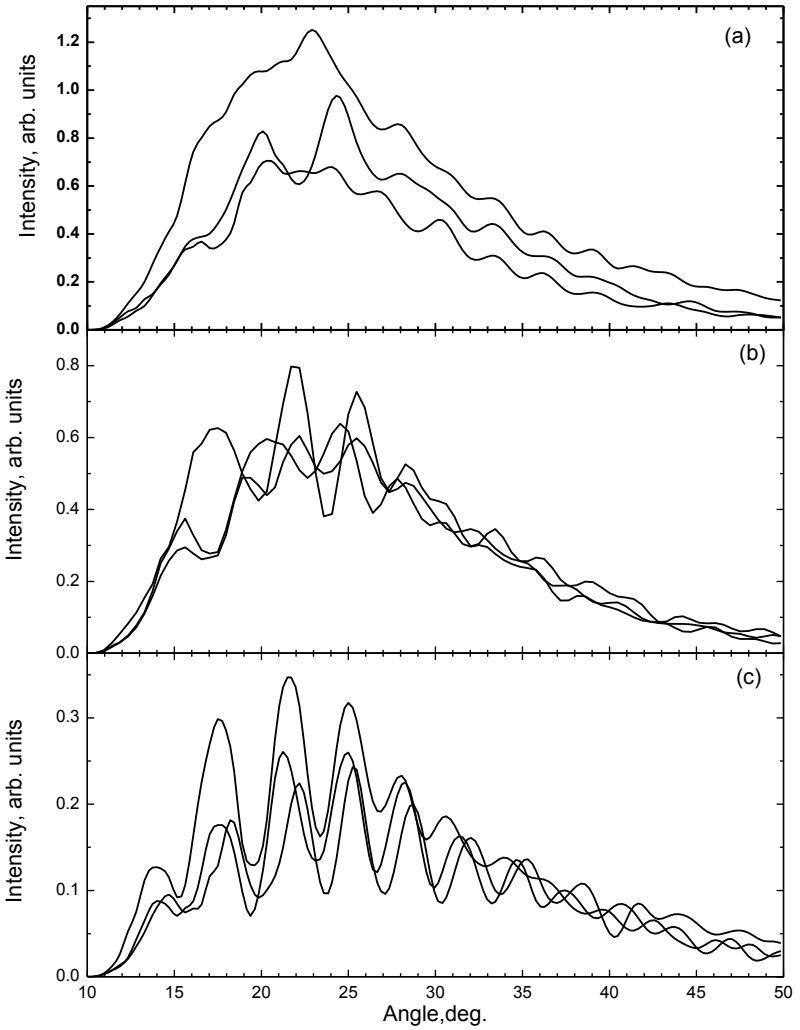


Figure 13. Weighted light-scattering profiles of RBCs in the course of the osmotic hemolysis at different time from the beginning: (a) initial; (b) 1 minutes; (c) 3 minutes.

## Red Blood Cells

Unlike a mononuclear cell, the red blood cell (RBC) has a homogeneous internal structure but a quite complex shape under normal conditions. In order to characterize the RBCs the biochemical treatment is used to spherize the RBCs and to solve the ILS problem

with Mie theory [9]. The shape of the RBC could be modeled by a biconcave disk. The mathematical formula that describes the shape of an RBC is a field of continuing research [34]. From the instrumental point of view we are ready to support the possible solution of the ILS problem for mature RBCs with maximally available data, i.e. regular and polarized LSPs measured with the SFC (Figure 12). The theoretical simulation of light scattering should be performed with the ADDA program code that means of usage of the nearest-neighbor interpolating within the LSP database for processing of experimental data. We are waiting for an appropriate optical model of a mature RBC to close the characterization problem for these cells. It is desirable that the high precision should be reached in determination of the RBC characteristics in order to be used for the analysis of different processes with these cells like a lysis or shape deformation.

Under normal conditions, a RBC maintains its volume, ionic composition, pH and membrane structure and integrity. When the RBC is placed in a hemolytic medium, it becomes unstable, and its volume changes. If the hemolysis is of the osmotic type, the osmotic gradient is established between intracellular and extracellular media, which causes water to flow into the cell leading to cell swelling. When the volume of the swollen cell exceeds a critical level, the integrity of their membranes is disrupted, allowing the hemoglobin to release into the external media. This is called hemolysis. The lysis observed in a large population of cells follows an S-shaped relation with time, reflecting the time-course of cell volume, the relation between volume and lysis, and the distribution of cell properties in the population. The capacity of RBCs to resist hemolysis can be modified by various pathologies and by physical, chemical or pharmacological treatments. Its study can thus yield relevant information for diagnostic purposes or fundamental researches.

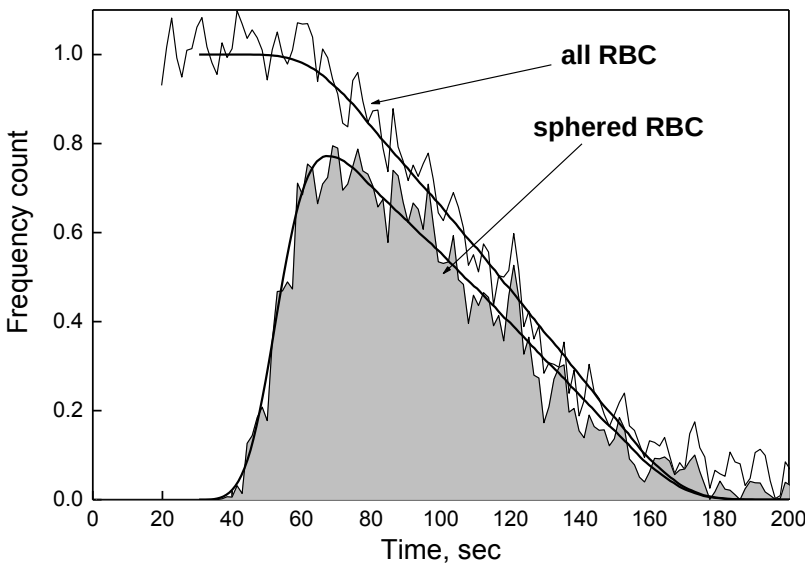


Figure 14. Kinetics of all and sphered RBC recognized from SFC traces.

A complete kinetic model of the hemolysis should contain three main elements: the first is a description of net ion fluxes; the second, cell volume as a function of solute content; and the third, a relationship between cell volume and lysis. There is much information and

substantial agreement about many aspects of RBC metabolism, membrane transport, pH control and volume regulation [35, 36, 37, 38, 39]. The non-ideal osmotic behavior of hemoglobin, the change in the net charge on impermeant cell ions with pH, and the kinetics and turnover rates of the main monovalent ion transporters are all well characterized, though in different, often incomparable experimental conditions. Following these models, the physiological properties of erythrocytes can be derived from kinetic volume changes [40]. The last stage of hemolysis, the rupture of the erythrocyte membrane, can be modeled as a spontaneous process by a causal sequence of two thermally activated transitions [41, 42], resulting in the dependence of the membrane breakage rate on the membrane tension. Recently, a complete mathematical model was developed that united all the above mentioned elements [43]. The model was applied successfully for the experimental study of osmotic hemolysis in isotonic solution of ammonium chloride ($NH_4Cl$) with the SCF technique. SFC was used to recognize sphered RBC from their LSPs (Figure 13) during the process. The obtained kinetics of all and sphered RBC (Figure 14) was compared with that of mathematical model [39] to evaluate the mean permeability and the mean strength (as well as their distributions over the cell population) of the RBC membrane.

## Conclusion

We review the state-of-the-art of light-scattering flow cytometry emphasizing the performance in solving the characterization problem for disperse systems using single-particle analysis. We conclude the angle-resolved light-scattering technique provides additional information in flow cytometry to allow us to solve the characterization problem. One possible instrumental solution is the scanning flow cytometry that can be used to measure the regular and polarized light-scattering profiles of individual particles. This technique allows for the determination of four characteristics of individual particles and two angles that describe the particle orientation. At present this is the maximal number of characteristics which can be retrieved with the light-scattering flow cytometry. Summarizing the results described in this chapter we would like to indicate the optical models which were successfully used in the solutions of the ILS problem: a sphere for polymer beads, spherized RDCs and blood microparticles; an oblate spheroid for blood platelets; a hemisphere capped cylinder for rod-like bacterial cells; a sphere with a cover for mononuclear cells. All particles were sized with the sub-diffraction precision. At present the hematology should take into the practice these methods to be available as a routine analysis in clinics in the same manner as RBC volume, RBC hemoglobin, and blood platelet volume.

Flow cytometry is currently the most powerful technology used in the characterization of individual wavelength-sized particles from light scattering. There is no other method that has yet demonstrated statistically proven analyses of disperse systems with sub-diffraction precision in particle sizing. At present the flow cytometry allows one to characterize not only a particle that can be modeled by a sphere, but also particles having rather complex shapes and internal structure. Of course there should be more developments in the characterization of complex particles, but this requires considerable efforts from scientists and especially mathematicians who might not be familiar with the needs of the biologist or life scientist. The life science disciplines have to absorb mathematics as much as possible. Following the

lessons of Immanuel Kant, "in any special doctrine of nature there can be only as much proper science as there is mathematics therein" [44]. These efforts should make the life science predictable satisfying the science definition in a short form that is "the development of a prediction procedure which has a calculable precision, even in the presence of perturbations".

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