Single-particle sizing from light scattering by spectral decomposition

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A Fourier transform was applied to size an individual spherical particle from an angular light-scattering pattern. The position of the peak in the amplitude spectrum has a strong correlation with the particle size. A linear equation retrieved from regression analysis of theoretically simulated patterns provides a relation between the particle size and the location of the amplitude spectrum's peak. The equation can be successfully applied to characterize particles of size parameters that range from 8 to 180 (corresponding to particle sizes that range from 1.2 to 27.2 μm at a wavelength of 0.633 μm). The precision of particle sizing depends on the refractive index and reaches a value of 60 nm within refractive-index region from 1.35 to 1.70. We have analyzed four samples of polystyrene microspheres with mean diameters of 1.9, 2.6, 3.0, and 4.2 μm and a sample of isovolumetrically sphered erythrocytes with a scanning flow cytometer to compare the accuracy of our new method with that of others.

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1. Introduction

Laser light-scattering techniques for the measurement of properties of single spherical particles are well established and routinely used in a variety of fields, ranging from ecology to medicine. Flow cytometry and phase Doppler anemometry have found various commercial applications.[1,2] Flow cytometry is in general applied for characterization of individual particles from light scattering and fluorescence. Phase Doppler anemometry is by far the most popular technique for simultaneous measurement of size and velocity of individual spherical particles.

Most optical particle sizing is based on Mie theory,[3] which refers to spherical, isotropic, and dielectrically homogeneous particles. A possible sizing strategy is to compute, for each particle with diameter d, index of refraction m, and illumination wavelength λ, scattering intensity I as a function of scattering angle θ and to compare the calculated angular scattering pattern, $I(\theta)$, with a measured angular scattering pattern.[4,5]

The optical and electronic designs of commercial flow cytometers provide measurements of light-scattering intensity integrated over fixed angular intervals (forward and side scattering). The inverse light-scattering problem was successfully solved for this optical setup by the 2ALS (Ref. 6) and the 3 × 2ALS (Ref. 7) methods. These methods are based on intensity maps calculated from Mie theory for a restricted region of particle size and refractive index. In particular, the volume distribution and hemoglobin concentration distribution of sphered erythrocytes were determined by Tycko *et al*. by the 2ALS method.[8] It should be noted that these methods entail a laborious calibration procedure for determination of absolute volume and hemoglobin concentration of individual red blood cells.

The high rate analysis in flow cytometry requires the development of suitable methods for solution of the inverse light-scattering problem. For such methods the real-time determination (i.e., in less than 1 ms) of the characteristics of an individual particle has great practical interest. Moreover, these methods should exclude calibration from mea-

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surements to simplify the instrumental characterization of individual particles from light scattering.

The inverse light-scattering problem can be solved by the measurement of an angular light-scattering pattern (from now on called the indicatrix) that is highly sensitive to particle morphology. Unfortunately the least-squares method that fits Mie-calculated and experimental indicatrices does not allow one to perform a real-time characterization. A parametric solution of the inverse light-scattering problem provides calibration-free and real-time characterization of individual spherical particles from light scattering.[9] The method can be applied for characterization of spherical particles with size parameters ranging from 6.6 to 100, whereas a phase-shift parameter must be in the range 0.2–10. The size and phase-shift parameters were defined as follows: size parameter $\alpha = m_0\pi d/\lambda$, where d is the particle diameter, m_0 is the refractive index of the medium, and λ is the wavelength of incident light; phase-shift parameter $\rho = 2\alpha(m - 1)$, where relative refractive index $m = m'/m_0$ and m' is the refractive index of the particle. Unfortunately, the method described in Ref. 9 is highly critical to the quality of the experimental data; i.e., it exhibits significant errors when it is processing noisy or distorted signals.

Traditionally, noisy experimental signals are processed by means of a spectral approach that assumes the formation of a Fourier spectrum of experimental signals. The spectral approach to solving the inverse light-scattering problem for individual homogeneous spheres was introduced by Ludlow and Everitt.[10] They used an expansion of the light-scattering indicatrix in the angular region from 0° to 180°, using a series of Gegenbauer functions. The cutoff point of the Gegenbauer spectrum was found to have a unique dependence on the size of the sphere. A Fourier transform was used by Min and Gomez to determine sizes of droplets with known indices of refraction.[11] They measured the light-scattering indicatrix with a photodiode array in angles from 9° to 18°. The array signal was processed with a fast Fourier transform (FFT) that generates two values, frequency and phase, which correspond to the number and angular positions of the scattering lobes. These two values provide an accurate indicator of the particle size. Godefroy and Adjouadi[12] proposed a flow-imaging experimental setup in which light scattering from individual particles was recorded by CCD camera in a two-dimensional light-scattering pattern. They used a two-dimensional FFT to estimate the sizes of spherical particles.

We propose a simple method for individual particle sizing with a parametric solution of the inverse light-scattering problem that uses Fourier parameters obtained from the indicatrix. A FFT was applied to size an individual spherical particle from indicatrices. The position of the peak in the amplitude spectrum has a strong correlation with the particle size. A linear equation retrieved from regression analysis of the theoretically simulated indicatrices provides a relation between the particle size and the location of the amplitude spectrum's peak. The equation can be successfully applied to characterize particles with size parameters that range from 8 to 180 (corresponding to particle sizes from 1.2 to 27.2 μm at a wavelength of 0.633 μm). The precision of particle sizing depends on the refractive index and reaches a value of 60 nm within the refractive-index region that ranges from 1.35 to 1.70. Four samples of polystyrene microspheres, with mean diameters of 1.9, 2.6, 3.0, and 4.2 μm, and a sample of isovolumetrically sphered erythrocytes were analyzed with a scanning flow cytometer (SFC).

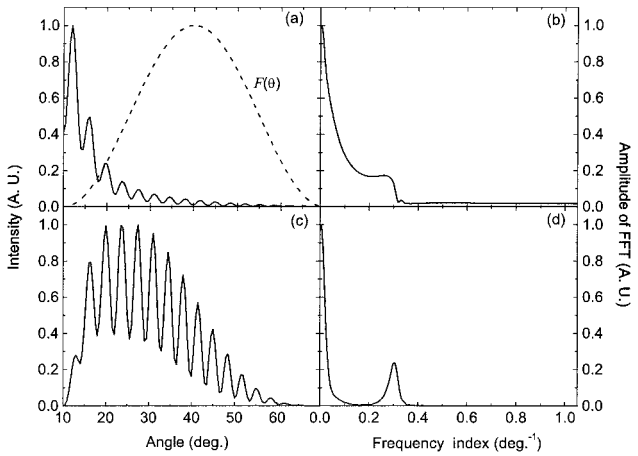

Fig. 1. (a) Light-scattering pattern with (b) spectrum of an individual spherical particle. Dashed curve, modifying function $F(\theta)$. (c) Light-scattering pattern modified by multiplication by function $F(\theta)$, (d) spectrum.

2. Size-Determination Algorithm

From a practical point of view we are interested in developing a noise-insensitive method for particle sizing that can be used with the SFC. The SFC allows the light-scattering indicatrices of individual particles to be measured within an angular region that ranges from 5 to 100 deg. The indicatrices of homogeneous spherical particles were calculated from Mie theory. The indicatrix of a particle with size parameter α of 53 and relative refractive index m of 1.16 is shown in Fig. 1(a). A FFT was applied to the calculated indicatrix and gave the amplitude spectrum shown in Fig. 1(b). The location of the steep shoulder in a spectrum can be related to the particle size. The analysis turned out to be problematic for determination of the location of this steep shoulder for small particles. To solve this problem we multiplied the indicatrix by a modifying function $F(\theta)$, shown in Fig. 1(a) by a dashed curve. The resultant modified indicatrix is presented in Fig. 1(c). Finally, the corresponding FFT spectrum of the modified indicatrix is presented in Fig. 1(d). There are two clear advantages in this modified spectrum: the steep shoulder is replaced by a clear peak and the width of the low-frequency part is greatly reduced.

It should be noted that some flexibility is available in the definition of function $F(\theta)$. The requirements

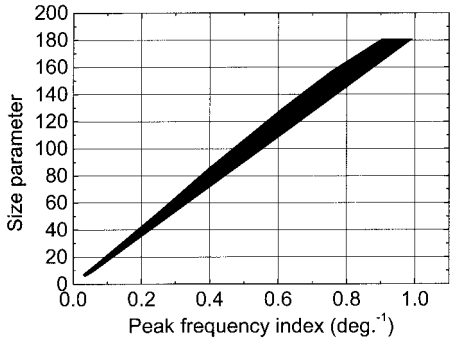

Fig. 2. Size parameters of particles with refractive indices n = 1.39, n = 1.49, n = 1.59, and n = 1.70 as a function of peak frequency index P_f.

are as follows: (1) $F(\theta)$ should be continuous and monotonic. (2) $F(\theta_l) = 0$, where θ_l is the lowest angle of the indicatrix. (3) $\Delta\theta \leq W \leq \Theta$, where W is the width of $F(\theta)$, $\Delta\theta$ is the distance between indicatrix minima, and Θ is the angular range of the indicatrix. The standard Hanning window procedure satisfies these requirements and greatly reduces the effects of the discontinuities at the beginning and the end of the sampling period. Here we have defined the function $F(\theta)$ in the following form:

$$F(\theta) = \sin^2\left(\pi \frac{\theta - \theta_l}{\theta_h - \theta_l}\right), \tag{1}$$

where θ_l and θ_h are the lowest and highest angles of the angular range of the indicatrix, respectively. The angular borders of the indicatrix are $\theta_l = 10°$ and $\theta_h = 70°$. To develop an algorithm that permits sizing of individual particles from the FFT spectrum of the modified indicatrix we have calculated indicatrices for particles with size parameters that range from 8 to 180 in increments of 1 (corresponding to size variations of 1.2–27.2 μm at a wavelength λ of 0.633 μm). Phase-shift parameter ρ was varied from 3 to 101 in increments of 2.8. The refractive indices of polystyrene beads and all biological cells are within the resultant range of refractive indices. The relation between size and the location of the peak in the frequency domain, P_F, for several refractive indices is shown in Fig. 2. The slope of the line is increased with increasing refractive index. P_f is measured in reciprocal degrees. A linear equation can be used to describe the dependence of size parameter α on P_F:

$$\alpha = kP_F, \tag{2}$$

where the coefficient $k = 191.32 \pm 0.04$ was obtained from linear regression. Equation (2) provides a standard deviation σ_α of 4.7 in the determination of size parameter that results in a mean relative error of 3.6% for particle sizing from the FFT spectrum of the modified indicatrix. Obviously, coefficient k depends on weighting function $F(\theta)$, whereas the standard deviation remains insensitive to a choice of the function.

The magnitude of the relative error is caused by a variation of refractive indices of particles within the bounds mentioned above. To improve the precision of size determination we have to take into account the effect of refractive index on the algorithm developed. We considered the following situations: arbitrary refractive index and conditionally definable refractive index.

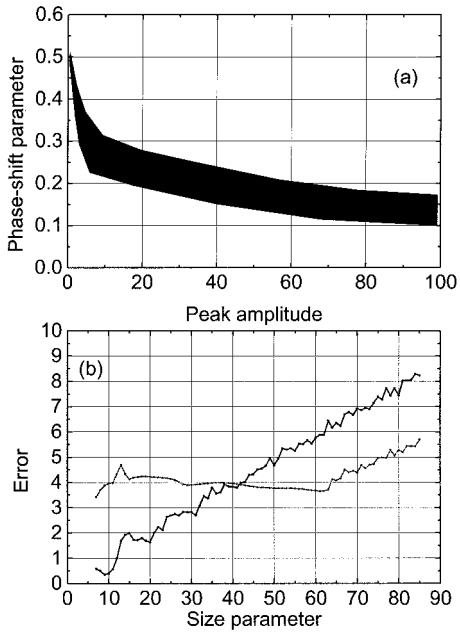

Fig. 3. (a) Scatterplot of the peak amplitude as a function of size and phase-shift parameters. (b) Maximal errors in calculation of size parameter from Eq. (2) (lighter curve) and from Eq. (3) (darker curve).

A. Effect of Refractive Index: Arbitrary Refractive Index

With the refractive index of a particle unknown, we have to choose additional parameters of the FFT spectrum that are sensitive to variation of the refractive index. Peak amplitude A_F of the peak in the normalized FFT spectrum has been chosen. This parameter has to reduce the effect of the refractive index on particle sizing from the FFT spectrum. A scatter plot of the peak amplitude is shown in Fig. 3(a) as a function of phase shift for various sizes within the region mentioned above. This scatterplot allowed us to choose the peak amplitude-sensitive term in the approximating equation. To obtain this approximating equation we applied a nonlinear fitting procedure to both the initial particle size and the size calculated from the tested approximating equations. A χ^2 test was used to minimize the residual standard error between initial and calculated sizes, and the final approximating equation corresponds to the minimum value of χ^2. The approximating equation that relates the parameters of size and the FFT spectrum, P_F and A_F, is

$$\alpha = k(1 + k_1/A_F)P_F + k_2, \tag{3}$$

where the coefficients $k = 163.79 \pm 0.09$, $k_1 = 0.02334 \pm 0.00009$, and $k_2 = 4.62 \pm 0.04$ were ob-

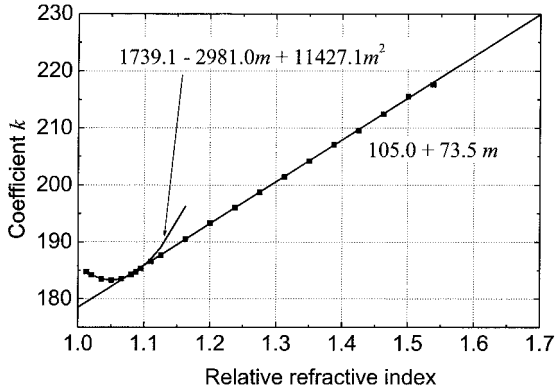

Fig. 4. Calculated dependency of coefficient k on relative refractive index (squares) and approximating equations for fitting them.

tained by nonlinear regression. Equation (3) provides a standard deviation σ_α of 2.3 for the determination of size from the FFT spectrum of the indicatrix. The size of a particle can be determined from the proposed algorithm with a precision of approximately 0.35 μm. The additional FFT parameter has allowed us to increase the precision of sizing by a factor of 2 compared with that provided by Eq. (2). However, the effect of refractive index is negligible for small sizes. The maximum systematic errors in calculation of size from Eqs. (2) and (3) are shown in Fig. 3(b) as a function of size by lighter and darker curves, respectively. The following conclusions were drawn from the functions: The effect of refractive index should be taken into account when the size parameter of an analyzed particle exceeds 40. If the size parameter is less than 40, Eq. (2) can be successfully applied for particle sizing with the spectral decomposition method. A size parameter of 40 corresponds to a 6-μm sphere suspended in water (refractive index, 1.333) and illuminated by red light (wavelength, 632.8 nm).

B. Effect of Refractive Index: Conditionally Definable Refractive Index

We analyzed the dependency of coefficient k [Eq. (2)] on the relative refractive indices of particles (Fig. 4). According to these data the region of relative refractive indices can be divided in two subregions: from 1.01 to 1.09 and from 1.09 to 1.50. The dependence of coefficient k on relative refractive index m can be approximated by a quadratic function in the range 1.01–1.09:

$$k = 1739.1 - 2981.0\ m + 1427.1\ m^2, \qquad (4)$$

with standard deviation $\sigma_k = 0.027$. In practice, coefficient k can be set at 184.2 without loss of accuracy. In the range of relative refractive index m from 1.09 to 1.50, k can be approximated by a linear equation:

$$k = 105.0 + 73.5\ m, \qquad (5)$$

with standard deviation $\sigma_k = 0.136$. Equations (4) and (5) applied to Eq. (2) give standard deviations σ_α of 0.282 and 0.382, respectively, for determination of size. The use of Eq. (2) with Eqs. (4) and (5) provides an error of approximately 0.06 μm for a size determination of spherical particles from light scattering. It should be mentioned that m can be set approximately; for instance, for polymer particles a relative refractive index of 1.2 gives suitable accuracy in sizing.

3. Experimental Equipment and Procedures

The experimental part of this study was carried out with the SFC, which allows measurement of the angular dependency of light-scattering intensity in a region that ranges from 5° to 100°. The design and basic principles of the SFC were described in detail elsewhere.[9,13] The light source was a He–Ne laser (Melles-Griot Model 05-LHP-151) with a wavelength of 0.6328 μm and an output power of approximately 10 mW. The indicatrices were measured in the range of 10°–70°. We applied a 512-point digital FFT to the experimental indicatrices.

Four samples of polystyrene microspheres (carboxylated polystyrene uniform latex microspheres, Duke Scientific), with mean diameters of 1.9, 2.6, 3.0, and 4.2 μm as specified by manufacturer, and a sample of isovolumetrically sphered erythrocytes have been measured in our experiments.

The method of sphering red cells before flow-cytometric analysis was the same as described in Ref. 14. Diluted whole blood (1/5000) was treated with 0.04% sodium dodecyl sulfate and 0.1% bovine serum albumin in isotonic phosphate buffer saline. The method permits isovolumetric sphering and is based on a change in membrane area while the osmolarity of the erythrocyte interior is preserved.

4. Results and Discussion

Latex particles are well suited for verification and comparison of methods developed for particle sizing. The parametric solution of the inverse light-scattering problem to determine particle sizes developed by Maltsev *et al.*[15] is based on measuring the distance between minima of the light-scattering indicatrix [flying light-scattering indicatrix (FLSI) method]. The approach to particle sizing presented in this paper improves the applicability of the parametric solution. The improvement consists in the development of a noise-insensitive method that utilizes spectral decomposition. The indicatrices of polystyrene beads measured with the SFC were processed by both methods. A typical experimental indicatrix, a modified indicatrix, and the spectral decomposition of a polystyrene bead are shown in Fig. 5. The FLSI method utilizes the distance between minima that occurs after a boundary angle of 15 deg. The indicatrix presented in Fig. 5(a) (open circles) gives a distance between minima $\Delta\varphi_1(15)$ of 7.19 deg. The spectral decomposition [Fig. 5(b)] gives the frequency index of 0.145 deg^{-1}, which corresponds to a distance between minima $\Delta\varphi$ of 6.89 deg. The distance between minima $\Delta\varphi$ is a superposition of distances between minima within the region ranging

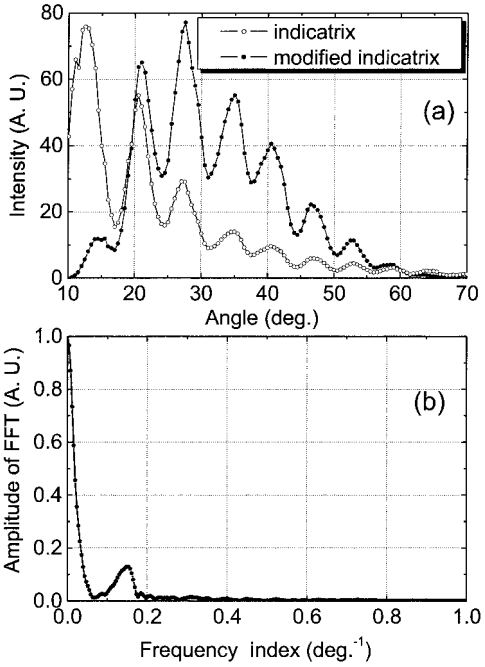

Fig. 5. (a) Typical experimental indicatrix of a polystyrene bead measured with a SFC and modified indicatrix. (b) Spectral decomposition.

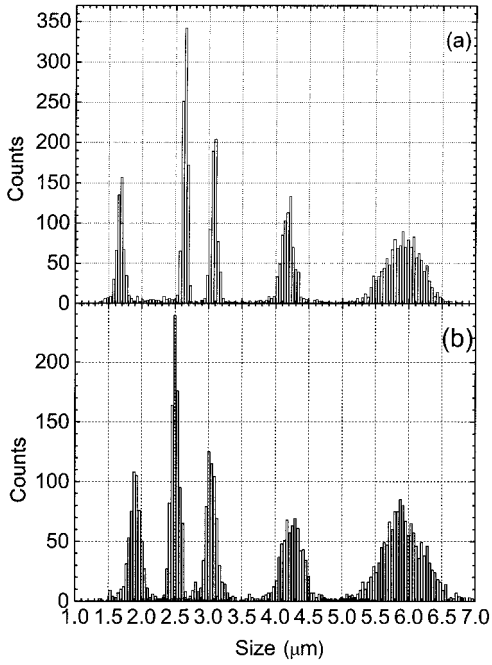

Fig. 6. Distribution of the sizes of polystyrene latex microspheres and sphered erythrocytes obtained in two ways: (a) based on Eq. (2) and (b) based on the FLSI method.

from 10 to 70 deg that reduces the effect of the boundary angle on the accuracy of individual particle sizing. The effect of the boundary angle when the FLSI method is used was discussed by Maltsev and Lopatin.[15] The experimental indicatrix shown in Fig. 5(a) was processed with a spectral decomposition method that gave the following sizes for the polymer bead: 4.19 μm from Eq. (2) and 4.23 μm from Eq. (5), assuming a relative refractive index of 1.2.

To demonstrate an effect of experimental noise on particle sizing of proposed method we added 10% white noise to the indicatrix presented in Fig. 5(a). The FLSI and spectral methods gave $\Delta\varphi_1(15) = 7.34$ deg and $\Delta\varphi = 6.87$ deg, respectively. The coincidence of $\Delta\varphi$ for noise-free and noisy indicatrices means that the new approach is noise insensitive, and we can expect improvement in the accuracy of individual particle sizing with spectral decomposition.

The size distributions for the samples of polystyrene microspheres and sphered erythrocytes were processed by the spectral and the FLSI methods, and the results are presented in Fig. 6 and Table 1. The mean sizes retrieved from Eq. (2) are less than 6 μm, with a size parameter of 39.6, which gives Eq. (2) an advantage over Eq. (3) [Fig. 3(b)]. This statement is in agreement with distribution parameters shown in Table 1. The distribution parameters retrieved from Eq. (2) are closer to manufacturer specifications than the parameters retrieved from Eq. (3). Nevertheless, the mean sizes of the distributions are in ranges of systematic errors introduced in Section 2 [Fig. 3(b)]. However, the difference between mean sizes of sphered erythrocytes for the two equations is negligible because the mean sizes are close to 6 μm, where the systematic errors are coincident. Indirect confirmation of the noise insensitivity of the spectral method resulted from fewer distribution widths retrieved from Eq. (2) than from the FLSI method (Table 1).

5. Conclusions

In this study we have improved the algorithm for the parametric solution of the inverse light-scattering

Table 1. Comparison of Equations for the Spectral Decomposition and FLSI Methods of Particle Sizing

	Equation (2)		Equation (3)		FLSI Method	
Number	Mean Diameter (μm)	Distribution Width (μm)	Mean Diameter (μm)	Distribution Width (μm)	Mean Diameter (μm)	Distribution Width (μm)
1	1.67	0.10	2.25	0.14	1.91	0.16
2	2.64	0.08	3.14	0.09	2.50	0.12
3	3.08	0.09	3.63	0.12	3.04	0.15
4	4.18	0.18	4.61	0.25	4.25	0.33
5	5.92	0.56	6.06	0.50	5.91	0.59

problem. The new method is insensitive to experimental noise, which results in more-precise sizing of individual spherical particles from light scattering. Additionally, the new method reduces a negative effect of the boundary angle on the precision of sizing. Spectral decomposition was demonstrated to be of practical significance because at present two experimental scanning flow cytometers are supported by this method for calibration-free real-time sizing. The spectral approach has the potential for use in the determination of refractive indices of spherical particles because of the sensitivity of the phase-shift parameter to the peak amplitude of the fast-Fourier-transform spectrum.

The new method should demonstrate advantages in the sizing of small particles, e.g., bacteria and spores, when the signal-to-noise ratio becomes relatively small. The spectral decomposition method plays an important role in the commercialization of the SFC, providing a stable and easy-to-use algorithm for different applications with this technique.

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References

1. M. R. Melamed, T. Lindmo, and M. L. Mendelsohn, eds., *Flow Cytometry and Sorting* (Wiley-Liss, New York, 1990).
2. H.-H. Qiu, W. Jia, C. T. Hsu, and M. Sommerfeld, "High accuracy optical particle sizing in phase-Doppler anemometry," Meas. Sci. Technol. **11,** 142–151 (2000).
3. C. F. Bohren and D. R. Huffman, *Absorption and Scattering of Light by Small Particles* (Wiley, New York, 1983).
4. M. Bartholdi, G. C. Salzman, R. D. Hiebert, and M. Kerker, "Differential light scattering photometer for rapid analysis of single particles in flow," Appl. Opt. **19,** 1573–1581 (1980).
5. I. K. Ludlow and P. H. Kaye, "A scanning diffractometer for the rapid analysis of microparticles and biological cells," J. Colloid Interface Sci. **69,** 571–589 (1979).
6. G. M. Quist and P. J. Wyatt, "Empirical solution to the inverse-scattering problem by the optical strip-map technique," J. Opt. Soc. Am. A **2,** 1979–1985 (1985).
7. V. P. Maltsev, "Estimation of morphological characteristics of single particles from light scattering data in flow cytometry," Russ. Chem. Bull. **43,** 1115–1124 (1994).
8. D. H. Tycko, M. H. Metz, E. A. Epstein, and A. Grinbaum, "Flow-cytometric light scattering measurement of red blood cell volume and hemoglobin concentration," Appl. Opt. **24,** 1355–1365 (1985).
9. V. P. Maltsev, "Scanning flow cytometry for individual particle analysis," Rev. Sci. Instrum. **71,** 243–255 (2000).
10. I. K. Ludlow and J. Everitt, "Application of Gegenbauer analysis to light scattering from spheres," Phys. Rev. E **5,** 2516–2526 (1995).
11. S. Min and A. Gomez, "High-resolution size measurement of single spherical particles with a fast Fourier transform of the angular scattering intensity," Appl. Opt. **35,** 4919–4926 (1996).
12. C. Godefroy and M. Adjouadi, "Particle sizing in a flow environment using light scattering patterns," Part. Part. Syst. Charact. **17,** 47–55 (2000).
13. J. T. Soini, A. V. Chernyshev, P. E. Hanninen, E. Soini, and V. P. Maltsev, "A new design of the flow cuvette and optical set-up for the scanning flow cytometer," Cytometry **31,** 78–84 (1998).
14. Y. R. Kim and L. Ornstein, "Isovolumetric sphering of erythrocytes for more accurate and precise cell volume measurement by flow cytometry," Cytometry **3,** 419–427 (1983).
15. V. P. Maltsev and V. N. Lopatin, "A parametric solution of the inverse light-scattering problem for individual spherical particles," Appl. Opt. **36,** 6102–6108 (1997).