# Light Scattering into Two Fixed Angles *vs*. Angle-Resolved Measurements for Characterization of Single Submicron Particles

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***Abstract*— Single particle identification and characterization based on scatter measurements is widely used in numerous biomedical applications. This scatter-based characterization approach implies a solution of the parametric inverse light-scattering (ILS) problem. The need for high-speed analysis limits the amount of collected scatter information and motivates maximum simplification of optical model of analyzed particles. We analyzed the capabilities and limitations of two existing approaches, based on measurement of either two scattering signals or angle-resolved patterns, applied to characterize single submicron particles. The standard flow cytometric approach is based on light scattering measurements into two fixed angles, forward and side scattering, which are further fitted by the Mie theory. We showed that corresponding ILS problem may have multiple solutions, and the procedure results in uncontrollable errors if the particle is not spherical. By contrast, angle-resolved scattering measurements have much larger information content at a cost of reduced analysis speed. This approach coupled with rigorous solution of ILS problem is shown to provide accurate identification and characterization of biological particles, including nonspherical ones.**

## I. Introduction

Owing to a wide implementation of light-scattering in numerous biomedical applications and studies of different types of biological particles, including blood cells, bacteria cells, etc., there is a growing interest in capabilities and limitations of light-scattering based technologies for particle analysis. Natural polydispersity of biological samples require implementation of single-particle measurement techniques for particle's detection and characterization, such as flow cytometry (FC). Standardly performed fluorescent labeling of specific cell subtypes does not allow one to detect and identify some cellular subpopulations or biological particles, i.e. for which specific fluorescent biomarkers are not available. Moreover, fluorescent analysis doesn't provide information on morphological properties of studied particles, sometimes significant as diagnostic parameters. This shifts FC researches of biological cells from fluorescent- to a scatter-based analysis, implying determination of particle characteristics, such as shape, size, and refractive index (RI), that naturally raises the question on reliability and accuracy of these estimations. At the same time, a strong need for high-speed analysis limits the amount of collected scatter information and tends to simplify scatterer optical models and assumptions, which underlie the solution of the parametric inverse light-scattering (ILS) problem.

Particularly, standard FC instruments measure only two scatter parameters, which are forward- (FSC) and side-scattering (SSC) intensities integrated in some angular ranges. This scatter data is obviously insufficient to both infer particle's characteristics and estimate their uncertainty, especially for complex biological objects. Still, FSC/SSC-based characterization approach is still widely applied for particle sizing, regardless of its limitations. By contrast, angle-resolved light-scatter measurements of individual particles have a potential to overcome at least some of highlighted shortcomings. Here we analyze the capabilities and limitations of these two particle characterization approaches. In particular, we demonstrate advantages of angle-resolved scattering measurements in identification and characterization of submicron biological particles based on the solution of ILS problem.

## II. Methods

### A. Light-scattering Measurements

Light-scattering measurements were performed with a Scanning Flow Cytometer (Cytonova Ltd Company, Novosibirsk, Russia), an advanced instrument capable of simultaneous measuring angle-resolved light-scattering patterns (LSPs) and standard flow cytometric forward scattering (FSC) and side scattering (SSC) signals for individual particles carried by flow through the optical measurement zone [1,2]. Within the Mueller-matrix formalism [3] for description of light scattering by a particle, the LSP, forward and side-scattering intensity (FSC/SSC) measured by Scanning Flow Cytometer (FSC) are expressed as:

$$I_{\text{th}}^{\text{LSP}}(\theta) = k_1 \int_0^{2\pi} \mathrm{d}\varphi \left[ S_{11}(\theta,\varphi) + S_{14}(\theta,\varphi) \right], \quad (1)$$

$$I^{\text{SSC/FSC}} = k_{\text{SSC/FSC}} \iint_{\Omega_{\text{SSC/FSC}}} \mathrm{d}\theta\, \mathrm{d}\varphi \sin\theta \times \left[ S_{11}(\theta,\varphi) - S_{12}(\theta,\varphi)\cos(2\varphi) - S_{13}(\theta,\varphi)\sin(2\varphi) \right], \quad (2)$$

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where $S_{ij}(\theta,\varphi)$ are elements of the Mueller matrix, $\theta$ and $\varphi$ are the polar and azimuthal scattering angles, $k_1$ and $k_{FSC/SSC}$ are the scaling coefficients, determined from calibration of the SFC, $\Omega_{FSC/SSC}$ – solid angles, determined by SFC detection optics. The LSP is measured at the wavelength of 405 nm in the polar angular range from 15° to 50° and the FSC and SSC are measured at the wavelength 488 nm. The FSC is integrated over annular aperture in angular range from 14.2° to 23°, SSC is integrated over circular aperture that is 90°±18.3° for both polar and azimuthal diametric angles respectively. Compared to SSC, FSC provides a wider range of detection of single spherical particles and, therefore, is used to trigger the electronics of SFC.

### *B. Particle Types*

We used polystyrene (PS) microspheres of five different sizes as reference particles to determine LSP, FSC, and SSC light acceptance angle ranges. Plasma membrane microparticles (MPs), a membrane-surrounded cell fragments derived from human blood plasma, were chosen for the analysis as representative biological particles, highly polydisperse by size (typically 0.1 – 1 μm), composition (i.e. RI), and shape (single spherical and nonspherical particles and their aggregates), and extensively studied by means of FC.

### *C. Light-scattering simulations*

We modelled reference particles and single spherical MPs as homogeneous spheres, described by size and RI. Scattering simulations were performed using Mie theory [3]. Small MPs aggregates, MP dimers, were modelled as bispheres, consisting of spheres of different diameters and equal effective RI, and thus were described by three morphological characteristics (two sizes and RI) and an auxiliary one (orientation angle in the flow of the SFC). Light scattering by a single dimer-like MP was simulated by the discrete dipole approximation (DDA) [4].

### *D. Inverse Light-scattering Problem Solution*

We employed two different approaches to solve the ILS problem, i.e. to characterize single particles from FC measurements. Standard FC scatter-based characterization approach implies fitting the FSC/SSC signals with the Mie theory, which we reduced to the solution of the system of two equations with two unknowns, diameter $d$ and RI $n$:

$$\begin{cases} I_{\text{exp}}^{\text{FSC}} = I_{\text{th}}^{\text{FSC}}(d,n), \\ I_{\text{exp}}^{\text{SSC}} = I_{\text{th}}^{\text{SSC}}(d,n), \end{cases} \tag{3}$$

using Levenberg–Marquardt method in multistart mode.

More advanced approach is based on simultaneous fitting of experimental LSPs, FSC and SSC signals by theoretical ones. Unfortunately, the FSC and SSC amplitudes can be used only for spherical particles, because otherwise they depend on another (azimuthal) orientation angle, in contrast to the LSP. The problem was transformed into the global minimization of the following weighted sum of squares:

$$S(\boldsymbol{\beta}) = \sum_i [w(\theta_i)]^2 \left[ \frac{I_{\text{exp}}^{\text{LSP}}(\theta_i) - \alpha I_{\text{th}}^{\text{LSP}}(\theta_i,\boldsymbol{\beta})}{\sigma_{\text{LSP}}} \right]^2 + \left[ \frac{I_{\text{exp}}^{\text{SSC}} - I_{\text{th}}^{\text{SSC}}(\boldsymbol{\beta})}{\sigma_{\text{SSC}}} \right]^2 + \left[ \frac{I_{\text{exp}}^{\text{FSC}} - I_{\text{th}}^{\text{FSC}}(\boldsymbol{\beta})}{\sigma_{\text{FSC}}} \right]^2, \tag{4}$$

where $\boldsymbol{\beta}$ is the vector of particle characteristics, $\theta_i$ is the polar scattering angle, $I_{\text{exp}}^{\text{LSP}}(\theta_i)$ and $I_{\text{th}}^{\text{LSP}}(\theta_i,\boldsymbol{\beta})$ are the intensities of experimental and theoretical LSP at angle $\theta_i$, respectively, $I_{\text{exp}}^{\text{FSC/SSC}}$ and $I_{\text{th}}^{\text{FSC/SSC}}(\boldsymbol{\beta})$ are experimental and theoretical FSC and SSC signals, respectively, $\sigma_{\text{LSP/FSC/SSC}}$ are experimental uncertainties of scatter measurements, $\alpha$ is the coefficient that compensates for the non-central particle trajectory in the flow cell of the SFC.

For spherical model we employed global optimization method DiRect [5], which provides an extensive search of global minima of $S(\boldsymbol{\beta})$ over the characteristics space confined by bounds $d \in [0.1, 1.2]$ μm, $n \in [1.35, 1.7]$, which amply cover the range of MPs. It is not feasible to apply direct fit to non-spherical particles due to much larger computational time required to simulate the LSP. Thus, we used the nearest-neighbour interpolation using the pre-calculated database of LSPs, described in details in [6].

Both applied algorithms not only find best-fit model characteristics by minimization of $S(\boldsymbol{\beta})$ but also calculate their mathematical expectations and uncertainties based on Bayesian method [6]. Among the two models applied to each MP the

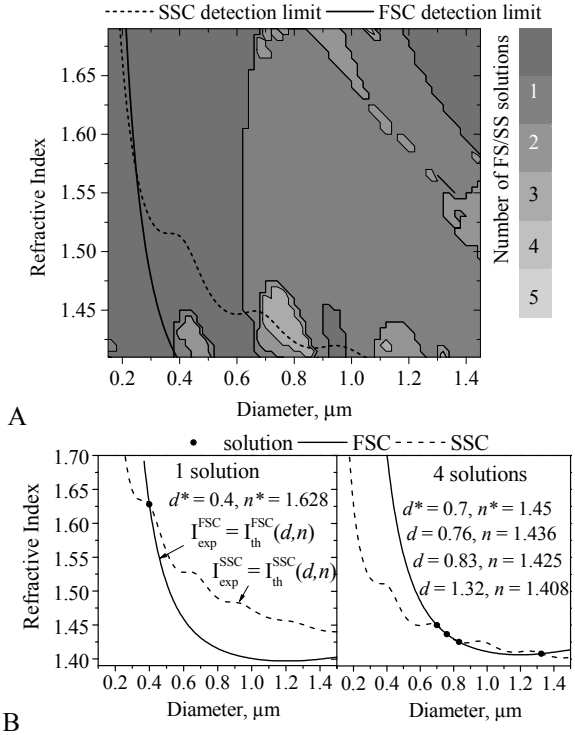


Fig. 1 (A) Evaluation of the solvability of ILS problem for spherical particles from FSC/SSC-scatter measurements in region of sizes and RI, which cover submicron PS microspheres and plasma MPs. (B) Typical examples, demonstrating uniqueness and multiplicity of solutions, including examples for PS microsphere (size 0.4 μm, RI = 1.628) and for MP (size 0.7 μm, RI = 1.45). Asterisk denotes true particle characteristics.

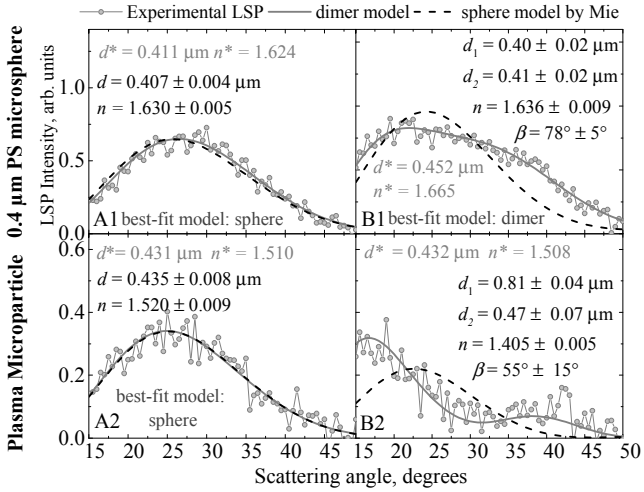


Fig. 2 Application of FSC/SSC- and angle-resolved LSP-based characterization approaches for particle characterization. The solution of ILS problem is demonstrated for single spherical 0.4 μm PS microsphere (A1), plasma MP (A2) (monomers) and their dimers (B1 and B2, respectively). For LSP-based characterization approach experimental and best-fit theoretical LSPs (for sphere and bisphere models) are shown, as well as estimates of particle characteristics, corresponding best-fit model (black font). Particle size and RI deduced from standard FSC/SSC measurements are also shown (gray font).

best one was chosen on a single-particle basis using the Bayesian information criterion.

## III. Results

The uniqueness of the solution of the ILS problem based on FSC and SSC measurements and described by Eq. (1), is known to depend on particular angular intervals, in which FC optics collects light scattered by a particle [7]. We analysed solvability of FSC/SSC scatter-based characterization approach in the range of sizes and RI, covering range of MPs and reference particles, and showed, that the uniqueness of the solution is provided only for the limited range of particles, some of which are undetectable by SFC (Fig. 1A). Typical examples demonstrating multiplicity of solutions are shown in Fig. 1B.

We applied both FSC/SSC- and angle-resolved LSP-based approaches to characterize measured PS microspheres and plasma MPs. For single spherical particles with detectable FSC and SSC signals both approaches led to similar results (Fig. 2). Size distributions of PS microspheres were also in perfect agreement with control measurements, performed by transmission electron microscopy (TEM). However, samples with PS microspheres and with MPs both contained particle's aggregates. As expected, incorrect application of Mie theory to characterize these detected events from FSC/SSC alone led to incorrect determination of particle's characteristics as demonstrated in Fig. 2. By contrast, LSP-based characterization approach allows not only to distinguish single- or dimer-like particles, that is clearly seen from (dis)agreement with Mie-theory in measured LSPs, but also to characterize them with known precision. Moreover, single spherical particles in polydisperse biological samples cannot be resolved from particles with other shapes by means of FSC or SSC, and require advanced angle-resolved scattering measurements.

## IV. Conclusion

Flow cytometric (FC) scatter-based single-particle analysis is the most widely applied light-scattering method for identification and characterization of different types of biological objects. A growing research interest to study different submicron-sized particles, including cell-derived membrane microparticles, extracellular vesicles, bacteria cells, etc. naturally raise question on challenges and opportunities of cytometry technologies for small particle characterization based on standardly performed forward- (FSC) and side-scattering (SSC) measurements.

In our work, we demonstrate that for single spherical microparticles simultaneous both size and RI can in principle be deduced from standard FSC/SSC measurements. However, the lack of scattering information significantly affects the solvability of the parametric ILS problem, limiting the range of characteristics in which the solution is unique. Moreover, there is no way to monitor uncertainty and reliability of performed estimates, as the deviation from the spherical shape silently introduces uncontrollable errors in determined characteristics. Rigorous analysis of the solution of the parametric ILS problem requires a greater amount of information to be extracted from scatter measurements. In particular, advanced angle-resolved scattering measurements enhance single particle analysis, including their accurate identification and characterization by shape, size, and RI.

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