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Elastic light-scattering measurements of single biological cells in an optical trap

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We have developed an instrument for determination of the angular light scattering of beads and biological cells. The instrument uses radiation pressure for levitation of particles inside a cuvette. The setup consists of two 780-nm diode lasers in a vertical double-beam trapping configuration. In the horizontal direction a weakly focused 633-nm probe beam is used to illuminate the trapped particle. One can detect scattered light over the range of from −150 to 150 deg with an angular resolution of 0.9 deg using an avalanche photodiode. With this setup light scattering from polystyrene beads was measured, and the obtained scattering patterns were compared with theoretical scattering patterns from Lorenz–Mie theory. The results show that the setup is stable, gives reproducible patterns, and qualitatively agrees with the calculations. Trapping of biological cells is more difficult than trapping of beads, because smaller forces result from smaller refractive indices. We present an angular scattering pattern measured from a human lymphocyte measured from 20 to 60 deg.

Key words: Angular light scattering, optical trapping, biological cell, microsphere.

1. Introduction

Light scattering is used for detection and characterization of small particles in various fields of science, for example, remote sensing of aerosols, detection of interstellar dust, quality control in industrial applications, and characterization of biological cells. Most of these applications use theoretical models based on idealized conditions: perfect spheres, perfect homogeneous particles, etc. Despite extensive research,[1,2] knowledge of light scattering of more complex structured particles is still limited.

During the last decade there has been increased attention to single-particle scattering. Bartholdi *et al.*[3] described a multiangle flow-through instrument for the detection of differential light scattering of polystyrene latex particles. Ashkin and Dziedzic[4,5] were the first to use optical trapping to manipulate and stabilize single particles in air for light-scattering measurements. In their study two laser beams were used to orient nonspherical particles. Forward-scattering patterns that originate from illumination by these lasers were recorded photographically. Misconi *et al.*[6] described an instrument in which a single beam levitated glass particles in air, and scattered light was detected in the range of 16–170 deg with an angular resolution of 2 deg. Guilloteau *et al.*[7] developed an optical trapping setup for the study of quasi-elastic light scattering. With this instrument one can obtain a decoupling of trapping and flat-field illumination by using the green and blue lines of one all-lines Ar^+ laser. Other researchers[8–13] have also shown the remarkable value of scattering experiments on trapped single particles for validation of scattering theories.

Much experimental research on forward and orthogonal scattering by single biological cells has been done in the field of flow cytometry.[3,14–19] Lofftus *et al.*[20] performed angle-dependent light-scattering measurements of single biological cells immobilized in silica aerogels and presented all sixteen Müller matrix elements of marine Dinoflagellates. Shapiro *et al.*[21] used the aerogel immobilization technique to investigate the coupled-dipole ap-

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proximation for modeling scattering by helical octopus sperm heads.

We describe an instrument that was designed for the measurement of angular light-scattering patterns of trapped cells and beads. The major difference from previous instruments is the use of optical trapping for suspension and immobilization of biological cells to perform angle-dependent light-scattering measurements. A second important difference is that suspension takes place in water. We implemented an opposing double-beam trapping configuration using diode lasers combined with a separate probe beam for homogeneous illumination of a particle in the trap. Furthermore, an almost 360-deg range of scattering angles ensures optimal information gathering and allows good comparison with theory. With this instrument it is now possible to obtain fundamental information about light scattering by single biological particles.

We have some direct and interesting applications for this setup. First, the experimental data can be used for checking theoretical models for scattering by cells.[14,16] Second, if one wants to improve flow cytometric differentiation of leukocytes,[22–24] the angular scattering patterns of the various cell populations have to be known. This instrument can provide these scattering patterns. Third, our group is also interested in the scattering patterns from polystyrene beads, which give remarkable results in flow cytometric measurements. With detailed data it might be possible to describe more accurately the Lissajous-like loops that appear in flow cytometric scatterplots.[25,26]

Our intention is to measure biological cells with this setup. However, we first determine the quality of the setup by measuring beads and comparing the results qualitatively with theoretical predictions. We obtained scattering patterns of 7.04-μm polystyrene beads over a large angular range and with large intensity changes within a small angular variation. Finally, we show the initial results of measuring the angular scattering pattern of a single human lymphocyte.

2. Apparatus

The optical trapping setup (based on the system of Bakker Schut[27]) of our instrument is shown in Fig. 1. Laser beams from two 780-nm, 100-mW diode lasers (Spectra Diode Laboratories 5311-G1) were collimated by diode-laser collimating lenses (Philips, type equivalent to RP036) and focused inside a cylindrical cuvette by two objectives (Leitz 32×, 0.4 NA). The waists of the elliptical foci are approximately 0.8 μm by 2.3 μm and located approximately 70 μm above each other. The cylindrical glass cuvette has an outer diameter of 7 mm and a height of 7 mm and is closed with a small microscope cover glass. The cuvette is polished to enable a clear view from all sides. The cuvette can be manipulated by an *XYZ* translation table (Oriel Model 18011) for transporting the particles to the two foci. Two

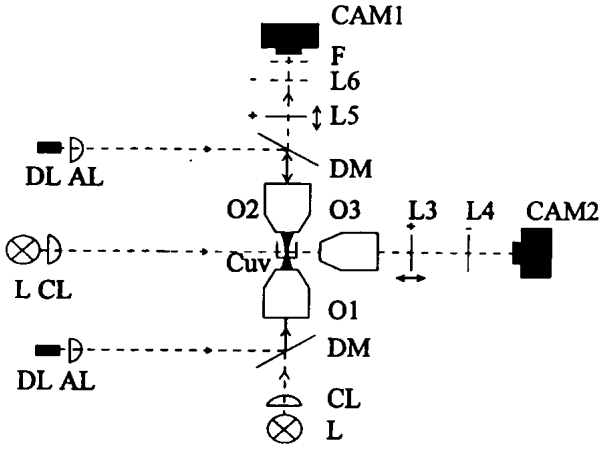

Fig. 1. Schematic setup of optical trapping and the microscope configuration: DL, diode laser; AL, aspherical diode-laser collimating lens; DM, dichroic mirror; O1. . .3, microscope objectives; Cuv, glass cuvette; L, lamp; CL, collimating lens; L3. .6, spherical lenses; F, short-pass filter; CAM1, 2, video cameras.

bright-field microscopes are incorporated in the setup to obtain visual control of the experiments in the horizontal and vertical directions. The dichroic mirrors (Ealing 35-5446) reflect the 780-nm light and transmit shorter wavelengths. This enables simultaneous trapping and imaging in the vertical direction. To decrease the intensity of the 780-nm trapping beam on the vertical camera, which leaks through the upper dichroic mirror, we inserted short-pass filters (Ealing 35-5388 and 35-5347).

Figure 2 shows the part of the setup that is used to illuminate the particle and to detect the scattered light. A chopped 10-mW Gaussian beam from a He–Ne laser (633 nm) is weakly focused with a 150-mm spherical lens, yielding a sufficiently uniform illumination pattern in the center of the cuvette (140-μm focal diameter). A motor-driven detection element can be rotated along a circular groove in the

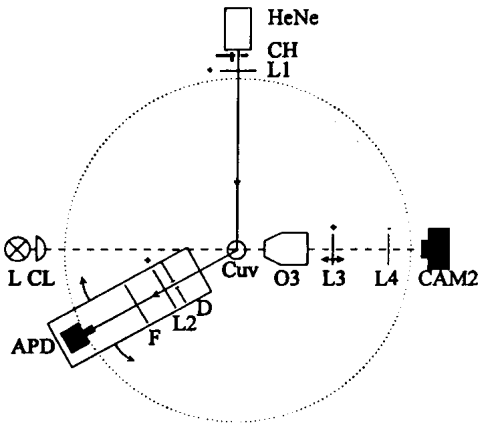

Fig. 2. Schematic setup of the scattering detection configuration: L, lamp; CL, collimating lens; Cuv, glass cuvette; O3, microscope objective; L1. . .4, spherical lenses; CAM2, video camera; HeNe, helium–neon gas laser; CH, chopper; D, diaphragm; F, short-pass filter; APD, avalanche photodiode.

optical table to measure the scattered light in the desired direction. This detection element consists of a diaphragm, which limits the detection cone, a positive lens, and a short-wavelength-pass filter. The diameter of the detection cone at the intersection point is calculated to be 120 μm. For the experiments shown here, the incident light is vertically polarized and the detector is not polarization selective, so the Mueller matrix element combination S11–S12 is measured.[1] Detection of the light is performed by an avalanche photodiode (EG&G C30902S). One can process the obtained photodiode signal using a phase-sensitive detection system. A PC-based data-acquisition system[28] is used to collect the data.

According to Guilloteau *et al.*,[29] the scattering patterns of particles larger than one third of the beam diameter show significant differences from the Lorenz–Mie theory (LMT)[30,31] and should be described with the generalized LMT,[32] which takes the exact beam shape into account. Our particles have a diameter that is only one twentieth of the beam diameter, so use of the ordinary LMT is permitted. The LMT implementation is based on the Bohren and Huffman program.[1]

3. Sample Preparation and Measurement Procedure

Particles were suspended in boiled (to remove dissolved air) and filtered (three times through a 0.22-μm Flowpore filter from ICN Biomedicals) water in a concentration of 2000/mL. The cuvette was filled with this solution, covered with a microscope cover glass, and sealed with glycerin a few hours prior to the experiments to allow sedimentation of the particles and obtain thermal equilibrium. This is necessary to simplify trapping of only one particle. Measurements were done with polystyrene spheres (7.04 ± 0.05 μm, Duke Scientific Corp.). The refractive indices of the beads and the water used in the calculations are 1.5774 and 1.3318, respectively, at 632.8 nm.[33] Human lymphocytes were isolated from peripheral blood from a healthy donor and suspended into a phosphate-buffered saline solution.[34] To reduce sticking of the cells and beads to glass surfaces,[35] Pluronic F127 (Molecular Probes, P1527) was added until a concentration of 1 mg/mL was reached.

An elaborate alignment procedure was performed prior to each measurement to certify correct measurements. The measurement procedures starts with trapping of a particle, followed by lowering the cuvette approximately 0.5 mm (to minimize the reflections of the probe beam on the bottom of the cuvette). Then angular measurements can be carried out. When high scattered intensities are measured, neutral density filters are inserted into the probe beam. To be able to match separate sections correctly, a small part of the scattering pattern is measured twice: once with and once without neutral density filters.

4. Results

Polystyrene beads can be readily trapped with this setup. The most stable configuration for 7.04-μm beads proved to be a double trap with a distance of approximately 70 μm between the two foci. A simple test of the lateral forces involved is the determination of maximum speed of the horizontally moving cuvette at which the bead remains trapped. The friction forces exerted by the liquid can be approximated with Stokes law. The measured maximum speed of a 7.04-μm polystyrene bead exceeds 200 μm/s, which indicates a lateral force larger than 13 pN. This value is in the typical range of forces that can be calculated using theoretical models; see, for example, Refs. 10 and 36.

The light-scattering pattern of a 7.04-μm bead is shown in Fig. 3. For comparison we inserted a calculated curve from the LMT using the size of 7.04 μm provided by the manufacturer and a refractive index of 1.5774. Although the two curves look similar, quantitative differences are observed. For example, the number of lobes per angle is different, indicating a difference in the size parameter value.

The stability of the trap was tested by fixing the detector at two angular positions and measuring the photodiode signal as a function of time. Recordings of the detector signal at the top of a scattering lobe and on a steep part of the curve both show small fluctuations over the observation period of 100 s. The amplitudes of the fluctuations for both recordings are the same, indicating a negligible fluctuation in angular scattering patterns. The fluctuations originate from electronic noise or Brownian motion of the bead.

Reproducibility was tested by repeated measure-

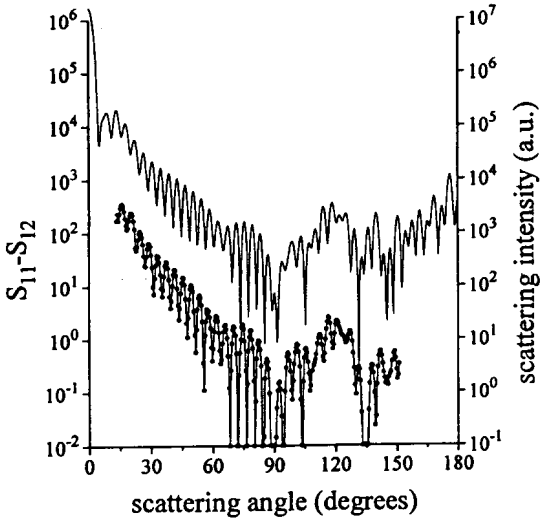

Fig. 3. Measured scattering curve of a 7.04 ± 0.05-μm bead (—●—, right axis) compared with a calculated curve (left axis). The calculated curve was obtained from the LMT (using a diameter of 7.04 μm, a refractive-index bead of 1.5774, and the refractive index of water of 1.3318) with integration over the detection cone.

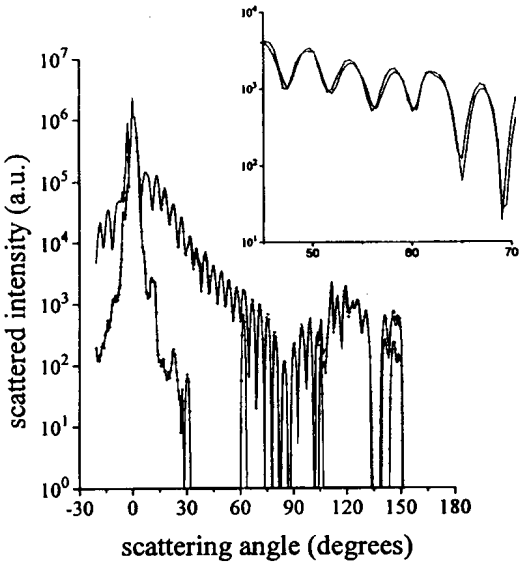

Fig. 4. Reproducibility test of the setup by repeated measurement of the same bead. The first (—●—) and second (——) measurements (after 30 min) yield almost the same curve. The inset shows even more clearly the close resemblance of the measured curves. The background measurement is indicated by —◇—.

ments of the same bead. In Fig. 4 two typical curves are shown. Measurement of the first curve took approximately 15 min. After a delay of 30 min, the second curve was measured using the same bead. As can be judged from the inset in Fig. 4, the reproducibility is excellent. A background measurement (a measurement without a bead in the trap) is also included in Fig. 4, which shows that background signals can be neglected from approximately 5 deg and up. At certain angles (62, 106, and 147 deg) background and signal levels are of the same order, but the background is still significantly lower. These high background levels are due to reflections in the setup. The scattering pattern of the bead shows large differences over a relatively small change in angle: a factor of almost 1000 for a change in angle of 2.25 deg in the region around 72 deg. These deep minima can be measured only for single particles, because for bulk measurements they are averaged out by the size distribution of the particles.[1] This indicates that the detection cone is chosen small enough to measure the scattering pattern accurately, and a comparison of measured and calculated curves is allowed.

Compared to polystyrene beads, trapping of biological cells is more difficult because of the smaller forces involved.[27] The experiment for determination of lateral forces for human lymphocytes showed a maximum speed of 35 μm/s. This means that the lateral forces are approximately 2.6 pN, at least five times weaker than that of the 7.04-μm polystyrene beads. The weaker forces are due mainly to the smaller refractive indices (relative refractive index of approximately 1.05). We observed a substantial influence of the microscope lamp that was used for illumination of the particles in the trap. The heat developed at maximum lamp intensity caused a convection flow inside the cuvette, which destabilized the trap resulting in loss of the trapped particle. The average time a cell remained in a trap (without a lamp) was approximately 20 min, which was hardly enough for our measurements. Another problem was the fact that cells stick to an uncoated glass cuvette and cannot be levitated easily. Sticking of the cells was reduced by using Pluronic: we succeeded in levitation of one in three cells.

Figure 5 shows a measurement of a trapped lymphocyte. We obtained an oscillating curve with fairly large intensity changes in the range from 20 to 60 deg. In this experiment we increased the detection cone angle to 3 deg, because signal levels were low. We observed fluctuations in the signal from the lymphocyte at each scattering angle. This instability is probably due to Brownian motion (rotation) of the cell. With an integration time of 10 s, we reduced the fluctuations to less than 5%.

5. Discussion

We have shown that light-scattering patterns of single biological cells can be measured using a double-beam trapping setup and a separate probe beam for illumination. We obtained patterns from polystyrene beads with a large intensity change within a small change of detection angle. With our current instrument a large detection angle range is possible: scattering angles from 150 to −150 deg. The results for polystyrene beads are highly reproducible.

It is important to realize the high sensitivity of the scattering for changes in refractive index and size. The observed differences between calculated and measured scattering curves (Fig. 3) can easily be explained by small size and refractive-index differ-

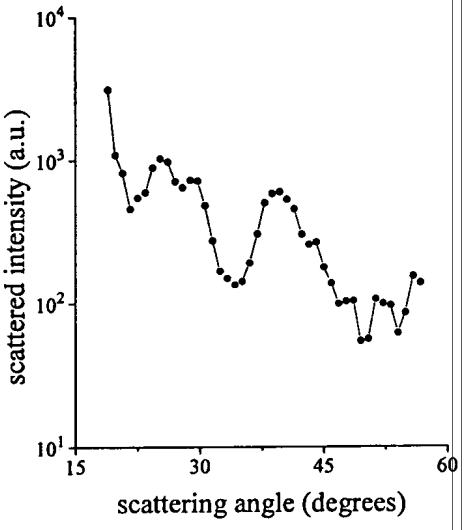

Fig. 5. Measured scattering curve of an optically trapped human lymphocyte in phosphate-buffered saline.

ences. To show this extreme sensitivity we calculated the three curves shown in Fig. 6. Differences in size of only 0.3% (equal to 0.4 times the standard deviation in the size of beads specified by the supplier) lead to large changes of the details of the curve: compare curve A with curve B. Similar changes of, for example, 0.6% in refractive index, lead to significant effects: compare curve A with curve C. The refractive index is temperature dependent: the change of the refractive index of water is −0.11% for a temperature change from 15 to 30 °C.[37] For polystyrene we estimated the change of refractive index with temperature from bulk cubic expansion coefficients and concluded that it was negligible. We do not expect large changes in scattering pattern because of temperature changes during the experiments, which we estimate to be a few degrees celsius at the most.

We used a polymer with hydrophobic and hydrophilic segments (Pluronic) to decrease sticking of polystyrene beads and cells to the surface of the glass cuvette. The change in refractive index is approximately +0.12% compared with plain phosphate-buffered saline. At present we do not know the influence of this polymer on the scattering properties of the particles. These effects can be investigated with our instrument, for example, by slowly adding polymer to the solution while continuously measuring the scattering pattern of a trapped particle.

The fluctuations of the signal from a trapped cell may be due to rotation of the cell caused by Brownian motion. Rotation may also be induced by misaligned trapping beams (caused by vertical displacement of the cuvette) giving rise to so-called hysteresis loops. Two beam traps can be misaligned in such a way that particles move in loops: moving upward by one beam to a point at which the forces of the other beam move the particle sideward and down again, forcing the particle into a closed loop. Another source of instability is the convection flow inside the cuvette that is generated by absorption of light by water, glass, or dirt particles. These problems might be solved by using higher trapping forces induced by either increased laser power or a changed beam configuration.

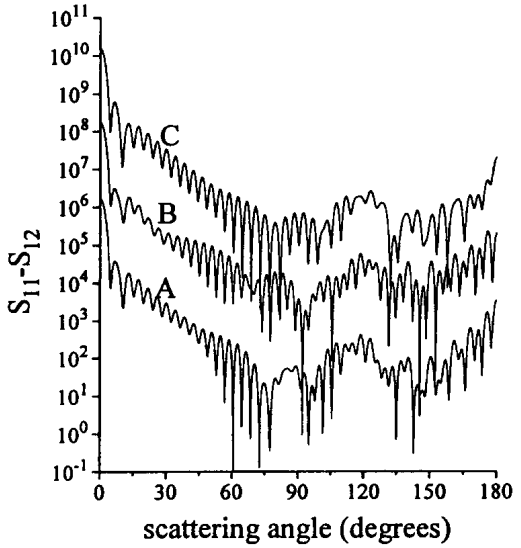

Fig. 6. Lorenz–Mie theoretical scattering curves for three slightly different beads: Curve A, sphere with a diameter of 7.04 μm and a refractive index of 1.5874 in water (n = 1.3318); curve B, slightly (0.3%) smaller sphere of 7.02 μm; the values were multiplied by 100 to separate the curves; curve C, sphere with a slightly (0.6%) larger refractive index, n = 1.5974; the values were multiplied by 10,000 to separate the curves. Note the large differences in the details of the curves.

Some estimates about the complete angular scattering pattern of lymphocytes can be made using information from flow cytometric measurements. From the forward light-scattering channel (which measures mainly the first scattering lobe: 0.5–3 deg) we know that lymphocytes have an approximately two times higher average signal compared with the 7.04-μm beads. In the perpendicular direction the flow cytometer can be used to measure scattered light integrated over a solid angle (half-angle of 26.8 deg) and the average signal from beads is a factor of approximately 7 higher than that of lymphocytes. From this information we expect the global pattern of a lymphocyte to be twice as high at small scattering angles and, on the average, seven times smaller at angles around 90 deg. Precise absolute measurements have to be performed yet. We can also make some estimates for the number of minima that could be expected in the scattering pattern of a lymphocyte. If we approximate the cell by a perfect sphere we can calculate the size parameter using average values for the actual size and the refractive index. With a diameter of 8 μm and a relative refractive index of 1.05 we expect the number of minima in the scattering pattern of Fig. 5 to be approximately 12. Obviously the measured pattern shows some irregular minima from which no definite number can be derived. This may be due to the internal structure and aspherical form of the cell, which is not taken into account in the LMT.

This instrument can be used to investigate various interesting biological problems. An example would be a study of the still unexplained depolarization effect that is exclusively observed with eosinophilic granulocytes in flow cytometric light-scattering measurements. Complete Mueller matrix measurements for a large angular range of (biological) particles are now feasible. Also dynamic light-scattering studies with single cells could yield new biologically relevant information.

6. Conclusions

We have built an instrument that is capable of measuring, over a large angular range, light-scattering patterns of micrometer-sized particles in water. The results for polystyrene beads are highly reproducible. Although the current stability problems limit measurement times, trapping and measuring human lymphocytes can be done.

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