

FIRST MEASUREMENT OF SIZE, REFRACTIVE INDEX, DEPOLARIZATION AND FLUORESCENCE OF PHYTOPLANKTON CELLS BY LASER SCANNING FLOW CYTOMETRY

*Luca Fiorani¹, Roberta Fantoni¹, Antonia Lai¹, Antonio Palucci¹, Konstantin A. Semyanov²,
Maria Sighicelli¹, Valeria Spizzichino¹ and Peter A. Tarasov²*

1. Laser Applications Section, ENEA, 45 Fermi Street, 00044 Frascati, Italy;
[fiorani\(at\)frascati.enea.it](mailto:fiorani(at)frascati.enea.it)
2. Institute of Chemical Kinetics and Combustion, SB RAS, 3 Institutskaya Street,
630090 Novosibirsk, Russia

ABSTRACT

A new laser flow cytometer, based on a laser source of polarized light irradiating microscopic particles, has been developed. The unpolarized and linearly polarized scattered light, as well as the side emitted light in different spectral bands, are measured, allowing the simultaneous and real-time determination of size, refractive index, depolarization and fluorescence of each particle. The tests with aqueous samples of polystyrene spheres, fluorescent or non fluorescent, and phytoplankton cells demonstrate that the system is able to retrieve size and refractive index with an accuracy of 1% and that the depolarization and fluorescence measurements allows the classification of particles otherwise indistinguishable.

INTRODUCTION

The simultaneous measurement of size and refractive index of microscopic particles by laser systems is based on the Mie theory that describes the light scattering by dielectric spheres (1). In general, the instruments based on this technique are named laser particle counters (2) and laser flow cytometers (3), if the particles are suspended in air and water, respectively. The great interest for the fast classification of microscopic particles in liquid suspensions (marine phytoplankton, blood cells, etc.) favored the broad diffusion of commercial systems based on laser flow cytometry (LFC).

In LFC, the particle flow and the laser beam are orthogonal. When a particle crosses the beam, the radiation is scattered with an angular distribution that depends on size and refractive index. In general, in the commercial systems only the forward scattering (FSC) and the side scattering (SSC) are measured, without retrieving size and refractive index. Often, the particle fluorescence is detected in some spectral bands. The forward scattering is associated with the particle size (4), while the side scattering is dominated by refractive effects (5). Although they are simplifications, this approach allows the fast classification of cells both in oceanographic (6) and clinical (7) fields, especially if the cells are marked with monoclonal antibodies conjugated to fluorescent dyes.

Typically, LFC allows one to simultaneously acquire 5 parameters: forward scattering, side scattering and fluorescence in 3 spectral bands. The counting rate can exceed 10 thousand particles per second. Among the advantages of LFC we recall the easy sample preparation, the absence of chemical treatment and the possibility of in situ measurements.

Recently, it has been realized CLASS, a laser flow cytometer for the characterization of phytoplankton cells, at the laboratories of Frascati of the Laser Application Section (LAS) of the Italian Agency for New Technologies, Energy and the Environment (ENEA), in collaboration with the Institute of Chemical Kinetics and Combustion (ICKC) of the Siberian Branch of the Russian Academy of Science (SB RAS).

Taking into account that:

- phytoplankton cells can be discriminated not only by size and refractive index, but also via shape and pigmentation,
- shape and pigmentation can be observed thanks to scattering depolarization and fluorescent emission, respectively,

it has been followed a research line allowing one to reach the objective consisting in the simultaneous measurement of size, refractive index, depolarization and fluorescence of microscopic particles.

To this respect, LAS and ICKC carry out complementary research from more than a decade: LAS developed local and remote sensors based on laser induced fluorescence (LIF) for bio-optical measurements of natural waters (8) and ICKC demonstrated the first example of laser scanning flow cytometry (LSFC) (9), able to retrieve size and refractive index. Both research lines resulted in prototype patents (10,11). Moreover, one of the authors used polarizing optics in the laser radar realized during his PhD thesis (12).

Eventually, combining LIF, LSFC and polarizing optics it has been developed CLASS, an innovative system able to simultaneously measure size, refractive index, depolarization and fluorescence of microscopic particles in liquid suspension.

INSTRUMENTS AND METHODS

Although CLASS represents the first application of LSFC to phytoplankton cells (13), the more innovative part of the system consist of the measurement of depolarization of microscopic particles. In order to carry it out, it has been necessary to use a diode laser with a high polarization ratio (100:1). Moreover, that source is characterized by large power and short wavelength, allowing the system to have good sensitivity and to detect sub-micrometric particle (up to about half of the wavelength). Potentially, it is possible to increase the polarization ratio inserting in the beam the Glan-Taylor polarizer Thorlabs GT10 (clear aperture diameter: 10 mm, extinction ratio: 100,000:1). The linearly polarized beam, after having been bent by three mirrors in order to make compact the system, gets across a quarter-wave plate, going out circularly polarized. Later, after having been focused by a lens and having got across a mirror with hole, the beam coaxially incides on a flow of particle in liquid suspension that gets across the capillary (diameter: 254 μm) of a cuvette. In the interaction region (length less than 5 mm) the beam has small cross section (diameter around 30 μm FWHM) and thus the radiant energy flow is high. The light scattered by the particle is reflected towards the mirror with hole by the spherical mirror that forms the bottom of the cuvette. The radiation reflected by the mirror with hole, after having been bent by a mirror in order to make compact the system, is split in two parts by a non polarizing beam splitter. The transmitted part is detected by the photomultiplier PM1, after having got across a polarizer, the reflected one is detected as it is by the photomultiplier PM2. Beam splitter, polarizer and photomultipliers are mounted on an aluminum block allowing only the light coming from the mirror with hole to pass. A variable diaphragm performs the spatial filtering of the input beam.

It should be noted that, for each position of the particle along the capillary, it is detected only the light coming from a well defined scattering polar angle, because it is observed the radiation collimated by the spherical mirror. Consequently, by retrieving the position from the acquisition time of the signal, thanks to the measurement of the particle velocity and its transit time in a known point, it is possible to determine the scattering intensity as a function of the polar angle (indicatrix) in a wide interval (typically between 5° and 100°). Eventually, size and refractive index of the particles are retrieved from the indicatrix by an inversion method (14) of the Mie theory. The transit time of the particle in a known point is measured, as we will see, by observing the side scattering. The particle velocity is a previously calibrated function of the pressures in the hydrodynamic circuit, selected by the operator.

If the particles were spherical, the signals I1 e I2, at the output of the photomultipliers PM1 e PM2, respectively, would be the same (unless a constant factor, linked to the different efficiency of the

respective detection channels, which can be made equal to 1 adjusting the gain of the photomultipliers). If, conversely, the particles are not spherical, the depolarization:

$$\delta I = I_1 - I_2 \quad (1)$$

is not zero. Consequently, the depolarization measurement will allow us to discriminate spherical particles: this is particularly useful to classify phytoplankton cells.

In order to demonstrate it, let us calculate I_1 and I_2 using the Mueller formalism (15). The radiation that incides on the particle has left-hand circular polarization. The corresponding Stokes vector is:

$$V_i = \begin{bmatrix} 1 \\ 0 \\ 0 \\ -1 \end{bmatrix}. \quad (2)$$

The Mueller matrices relative to particle scattering, beam splitter and polarizer are, respectively:

$$S = \begin{bmatrix} S_{11} & S_{12} & S_{13} & S_{14} \\ S_{21} & S_{22} & S_{23} & S_{24} \\ S_{31} & S_{32} & S_{33} & S_{34} \\ S_{41} & S_{42} & S_{43} & S_{44} \end{bmatrix}, \quad (3)$$

where each element of S is a function of the polar (θ) and azimuthal (φ) scattering angles,

$$B = \begin{bmatrix} 1/2 & 0 & 0 & 0 \\ 0 & 1/2 & 0 & 0 \\ 0 & 0 & 1/2 & 0 \\ 0 & 0 & 0 & 1/2 \end{bmatrix} \quad (4)$$

and

$$P = \begin{bmatrix} 1 & 1 & 0 & 0 \\ 1 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \end{bmatrix}. \quad (5)$$

The Stokes vector of the photomultiplier is:

$$V_{PM} = [1 \ 0 \ 0 \ 0]. \quad (6)$$

Since the azimuthal angles in the laboratory system and in the particle system do not coincide, one has to apply the laboratory-particle transformation matrix:

$$M_\varphi = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & \cos(2\varphi) & \sin(2\varphi) & 0 \\ 0 & -\sin(2\varphi) & \cos(2\varphi) & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix} \quad (7)$$

the particle-laboratory transformation matrix:

$$M_{-\varphi} = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & \cos(2\varphi) & -\sin(2\varphi) & 0 \\ 0 & \sin(2\varphi) & \cos(2\varphi) & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix}. \quad (8)$$

Moreover, it is necessary to integrate over φ between 0 and 2π , because the reflectance of the spherical mirror of the cuvette is independent from the azimuthal angle. Eventually, we can write:

$$I_1 = k_1 \int_0^{2\pi} V_{PM} P B M_{-\varphi} S M_{\varphi} V_i d\varphi, \quad (9)$$

$$I_2 = k_2 \int_0^{2\pi} V_{PM} B M_{-\varphi} S M_{\varphi} V_i d\varphi, \quad (10)$$

where k_1 and k_2 are constant factors, linked to the efficiencies of the respective detection channels. Developing the calculus, we obtain:

$$I_1 = k_1 \int_0^{2\pi} [s_{11} - s_{14} + (s_{21} - s_{24}) \cos(2\varphi) - (s_{31} - s_{34}) \sin(2\varphi)] d\varphi, \quad (11)$$

$$I_2 = k_2 \int_0^{2\pi} (s_{11} - s_{14}) d\varphi. \quad (12)$$

For spherical particles:

$$I_1 = k_1 \int_0^{2\pi} s_{11} d\varphi, \quad (13)$$

$$I_2 = k_2 \int_0^{2\pi} s_{11} d\varphi. \quad (14)$$

After having introduced spherical particles in the experimental system and having tuned the gain of photomultipliers PM1 and PM2 so that:

$$k_1 = k_2 = k, \quad (15)$$

we can write:

a) in general:
$$I_1 = k \int_0^{2\pi} [s_{11} - s_{14} + (s_{21} - s_{24}) \cos(2\varphi) - (s_{31} - s_{34}) \sin(2\varphi)] d\varphi, \quad (16)$$

$$I_2 = k \int_0^{2\pi} (s_{11} - s_{14}) d\varphi, \quad (17)$$

$$\delta I = k \int_0^{2\pi} [(s_{21} - s_{24}) \cos(2\varphi) - (s_{31} - s_{34}) \sin(2\varphi)] d\varphi, \quad (18)$$

b) for spherical particles:
$$I_1 = k \int_0^{2\pi} s_{11} d\varphi, \quad (19)$$

$$I_2 = k \int_0^{2\pi} s_{11} d\varphi, \quad (20)$$

$$\delta I = 0. \quad (21)$$

The system observes also the side radiation by a microscope objective. The light is split in three spectral zones by two dichroic mirrors. The reflection of the first dichroic mirror sends the side scattering to the photomultiplier PM₅ that measures the transit time of the particle in a known point and provide the trigger to the experiment. The photomultiplier is mounted on an aluminum block allowing only the light coming from the particle to pass. A variable diaphragm and an interference filter perform the spatial and spectral filtering, respectively, of the input beam. The second dichroic mirror:

- reflects the green radiation towards the photomultiplier PM₄ that measures the fluorescence of the particle around 530 nm, after the spectral filtering carried out by an interference filter,
- transmits the red radiation to the photomultiplier PM₃ PM₄ that measures the fluorescence of the particle around 680 nm, after the spectral filtering carried out by an interference filter.

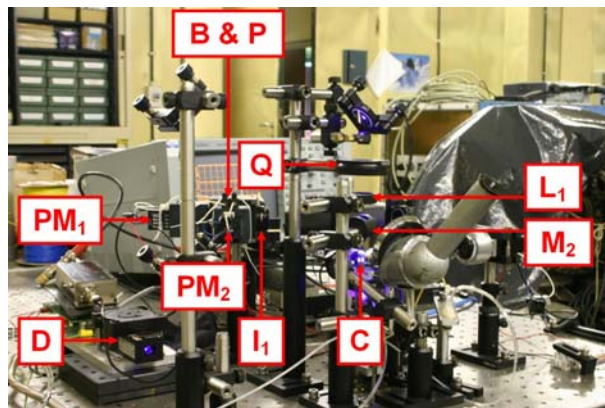


Figure 1: Picture of the system. The main elements are indicated with the naming convention followed in Table 1.

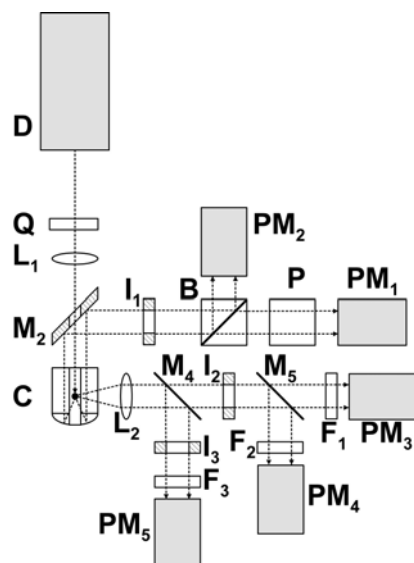


Figure 2: Optical scheme of the system. The elements are described in Table 1. The 3 bending mirrors M1 (inserted between D and Q) and the bending mirror M3 inserted between M2 and I1) are not influential and have not been indicated.

Table 1. Optical elements of the system. λ : wavelength, \varnothing : diameter, AOI: angle of incidence, R: reflectance, AR: anti reflection, f: focal length, h: height, NA: numerical aperture, T: transmittance, CA: clear aperture, BW: bandwidth.

Element	Description	Characteristics	Producer/Model
D	Diode laser	λ : 405 nm, power: 50 mW, polarization ratio: 100:1	μ LS/Lepton IV
M ₁	Broadband dielectric mirror	\varnothing : 25.4 mm, AOI: 45°, R>97% (400 – 750 nm)	Micos
Q	Zero order quarter-wave plate	λ : 405 nm, \varnothing : 12.7 mm, AR coating at 405 nm	Micos
L ₁	Plano-convex lens	f: 60 mm, R<3% (350 – 650 nm)	Thorlabs/LA1134-A
M ₂	Broadband dielectric mirror with hole	\varnothing : 25.4 mm, hole \varnothing : 1 mm, R>97% (400 – 750 nm)	Micos
C	Cuvette with spherical mirror	\varnothing : 5 mm, h: 5 mm	ICKC
M ₃	Broadband dielectric mirror	\varnothing : 25.4 mm, AOI: 0° – 45°, R>98% (400 – 800 nm)	Thorlabs/BB1-E02
I ₁	Variable iris diaphragm	\varnothing : 2 mm	Thorlabs/D12S
B	Non polarizing beam splitter cube	Side: 10 mm, T and R> 40% (400 – 600 nm)	Thorlabs/BS010
P	Glan-Thompson polarizer	CA \varnothing : 10 mm, extinction ratio 100,000:1	Thorlabs/GTH10M
PM ₁	Photomultiplier module	λ : 300 – 650 nm (peak: 420 nm), sensitivity: 4.3×10^4 A/W. Connected to the amplifier Femto HCA-10M-100K-C	Hamamatsu/H6780
PM ₂	Photomultiplier module	λ : 185 – 650 nm (peak: 420 nm), sensitivity: 4.3×10^4 A/W. Connected to the amplifier Analog Modules 352-1-B-1M	Hamamatsu/H6780-03
L ₂	Microscope objective	Magnification: 50 \times , NA: 0.55	Zeiss/LD EC Epiplan-Neofluar
M ₄	Dichroic mirror	\varnothing : 25.4 mm, AOI: 45°, R>90% (405 nm) T>70% (>435 nm)	Laser Components/630DRLP
I ₂	Variable iris diaphragm	\varnothing : 4 mm	Thorlabs/D12S
M ₅	Dichroic mirror	\varnothing : 25.4 mm, AOI: 45°, R>90% (460 – 620 nm) T>70% (>660 nm)	Laser Components/435DRLP
F ₁	Interference filter	\varnothing : 25.4 mm, T: 74% (680 nm), BW: 11 nm	Laser Components/680DF10
PM ₃	Photomultiplier module	λ : 300 – 900 nm (peak: 630 nm), sensitivity: 3.9×10^4 A/W. Connected to the amplifier Femto HCA-1M-1M-C	Hamamatsu/H6780-20
F ₂	Interference filter	\varnothing : 25.4 mm, T: 64% (530 nm), BW: 6 nm	Omega Optical/530BP5
PM ₄	Photomultiplier module	λ : 185 – 850 nm (peak: 400 nm), sensitivity: 3.0×10^4 A/W. Connected to the amplifier Femto HCA-1M-1M-C	Hamamatsu/H6780-04
I ₃	Variable iris diaphragm	\varnothing : 1 mm	Thorlabs/D12S
F ₃	Interference filter	\varnothing : 25.4 mm, T: 51% (402 nm), BW: 5 nm	Omega Optical/403BP5
PM ₅	Photomultiplier module	λ : 185 – 750 nm (peak: 420 nm), sensitivity: 5.2×10^5 A/W. Connected to the amplifier Analog Modules 352-1-B-1M	Hamamatsu/H7710-11

First of all, we observe that the system measures correctly size and refractive index of the spheres. In fact, the expected values: $6.0 \mu\text{m}$ and $1.61 - 1.62$ (13), respectively, are compatible with the measured values: $6.07 \pm 0.27 \mu\text{m}$ and 1.629 ± 0.022 , respectively (obtained by averaging the results of $6 \mu\text{m F}$ e $6 \mu\text{m NF}$), corresponding to relative errors of 1% in both cases. Moreover, as we expected, all the spheres have zero depolarization and the fluorescence is different from zero only for $6 \mu\text{m F}$.

As far as the particle classification is concerned, we note that, due to the large natural variability in size of the cells (observed at the microscope), the corresponding histogram is rather wide and it is impossible distinguish them from the spheres. Also for the refractive index an analogous argument holds: although the values for cells and spheres are significantly different, it exists a non negligible overlap in the histograms: a cell can be mistaken as a sphere and vice versa. The situation becomes better if the fluorescence is observed: the non fluorescing spheres are completely resolved. Unfortunately, still there can be confusion between fluorescent spheres and cells. Eventually, whenever the depolarization is taken into account, it is impossible to mistake cells and spheres.

These results are even more evident in the two-dimensional and three-dimensional scatter plots of Figure 6 e 7, respectively, and demonstrate how useful is the depolarization measurement for the accurate classification of microscopic particles: without it, it would have been impossible to fully distinguish cells and spheres.

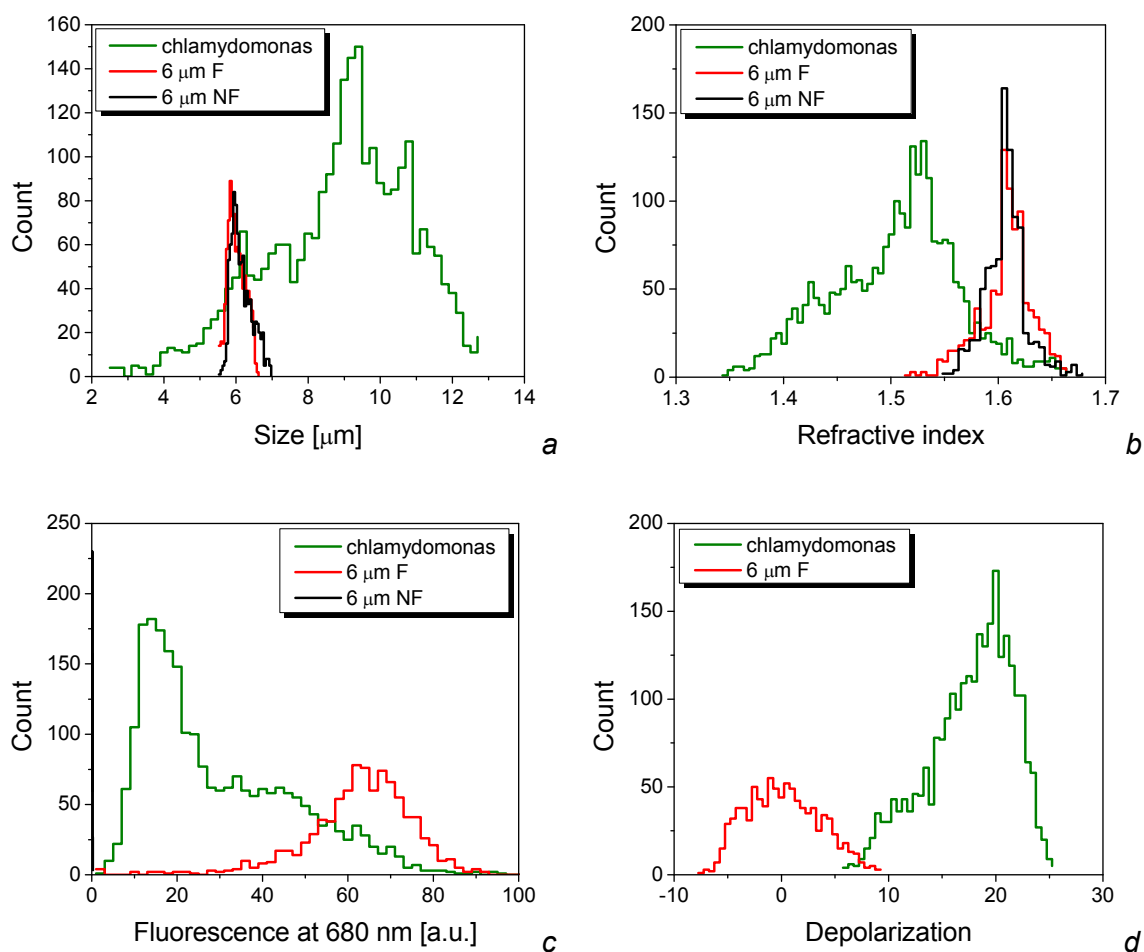


Figure 5: Histograms of: a) size, b) refractive index, c) fluorescence and d) depolarization obtained analyzing a sample of *Chlamydomonas reinhardtii* mixed with fluorescent and non fluorescent polystyrene spheres, having a $6 \mu\text{m}$ diameter.

Table 2: Measurements of size, refractive index, depolarization and fluorescence obtained analyzing a sample of *Chlamydomonas reinhardtii* mixed with fluorescent and non fluorescent polystyrene spheres, having a 6 μm diameter. M: mean, SD: standard deviation.

Particle	Number	Size (μm)		Refractive index		Depolarization (a.u.)		Fluorescence (a.u.)	
		M	SD	M	SD	M	SD	M	SD
Chlamydomonas	2627	8.79	1.97	1.526	0.059	17.4	4.0	26	19
6 μm F	924	6.00	0.24	1.629	0.025	0.0	3.5	61	13
6 μm NF	909	6.13	0.29	1.629	0.019	0.1	3.5	0	0

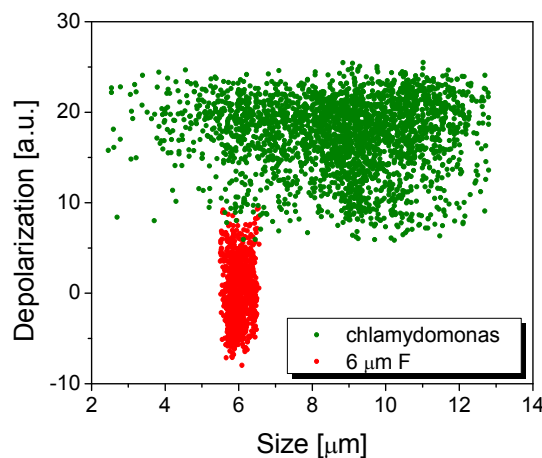


Figure 6: Two-dimensional scatter plot (size and depolarization) obtained analyzing a sample of *Chlamydomonas reinhardtii* mixed with fluorescent polystyrene spheres, having a 6 μm diameter.

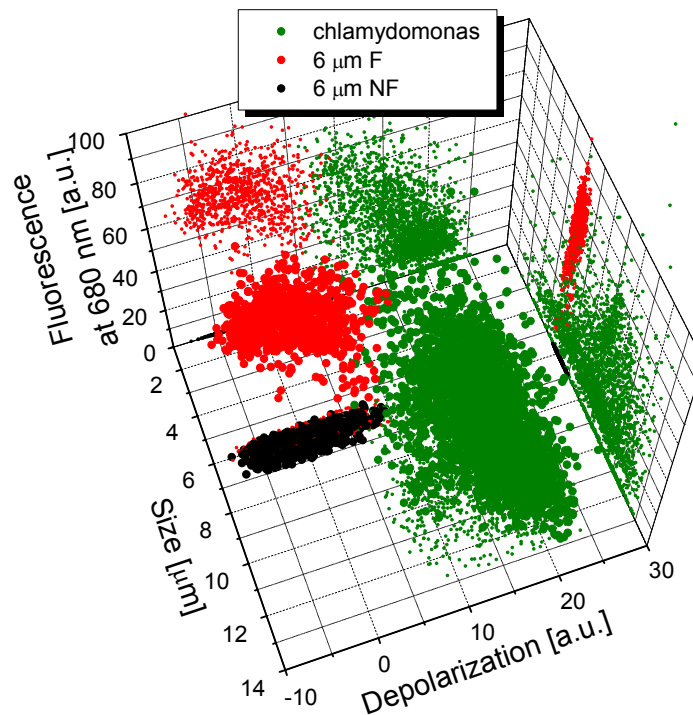


Figure 7: Three-dimensional scatter plot (size, depolarization and fluorescence) obtained analyzing a sample of *Chlamydomonas reinhardtii* mixed with fluorescent and non fluorescent polystyrene spheres, having a 6 μm diameter (the smaller points are the projections on the planes).

In order to give an example of the potentiality of the fluorescence observation in two spectral bands, it has been measured a sample prepared mixing green fluorescent and non fluorescent polystyrene spheres, having a 2 μm diameter (indicated as “2 μm F” and “2 μm NF”, respectively), with red fluorescent and non fluorescent polystyrene spheres, having a 6 μm diameter (indicated as “6 μm F” and “6 μm NF”, respectively). The three-dimensional scatter plot of Figure 8 demonstrates that CLASS resolves the four types of particles in well distinct clouds of points.

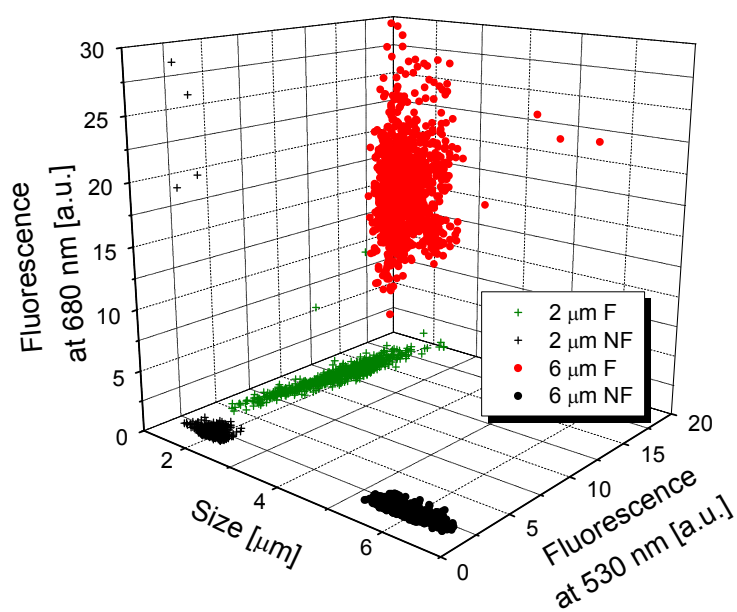


Figure 8: Three-dimensional scatter plot (size, fluorescence at 530 nm and fluorescence at 680 nm) obtained analyzing a sample prepared mixing green fluorescent and non fluorescent polystyrene spheres, having a 2 μm diameter, with red fluorescent and non fluorescent polystyrene spheres, having a 6 μm diameter.

CONCLUSIONS

A new laser flow cytometer (CLASS), has been developed. Its more relevant feature is the introduction of a polarized source and the detection of unpolarized and linearly polarized scattered light. Thanks to that innovation, simultaneous measurements of size, refractive index, depolarization and fluorescence of microscopic particles have been carried out. To our knowledge, they represent the first example of those results by laser flow cytometry.

The tests conducted on polystyrene spheres show that some thousands of particles can be analyzed in few minutes and indicate that the accuracy of CLASS in the retrieval of size and refractive index can exceed 1%.

CLASS has been applied to the experimental characterization of phytoplankton cells. Those researches confirm the role of fluorescence measurements and, more important, demonstrate the role of depolarization measurements in the classification of particles otherwise indistinguishable by size and refractive index determinations.

ACKNOWLEDGEMENTS

The authors are deeply grateful to P. Aristipini for technical drawings, R. Giovagnoli for mechanical parts, F. Barnaba and V. M. Nekrasov for system tests and P. Albertano for chlamydomonas samples. This work has been supported by the Italian Ministry of University and Research (MUR) in the frame of the project Microsystems for Hostile Environments (MIAO).

REFERENCES

- 1 Tsang L, J A Kong & K-H Ding, 2001. Scattering of Electromagnetic Waves: Theories and Applications (John Wiley & Sons, New York)
- 2 Provder T & J Texter, eds.,2004. Particle Sizing and Characterization (American Chemical Society, Washington)
- 3 Shapiro H M, 2003. Practical Flow Cytometry (John Wiley & Sons, New York)
- 4 Burger D E, J H Jett & P F Mullaney, 1982. Extraction of morphological features from biological models and cells by Fourier analysis of static light scatter measurements. Cytometry, 2, 327-336.
- 5 Salzman G C, P F Mullaney & B J Price, 1979. Light-scattering approaches to cell characterization. In: Flow Cytometry and Sorting, M R Melamed, T Lindmo & M Mendelsohn, editors (John Wiley & Sons, New York)
- 6 Dubelaar G B J & R R Jonker, 2000. Flow cytometry as a tool for the study of phytoplankton. Sci. Mar. 64: 135-156.
- 7 O'Leary T J, 1998. Flow cytometry in diagnostic cytology. Diagn. Cytopathol. 18: 41-46.
- 8 Fiorani L & A Palucci, 2006. Local and remote laser sensing of bio-optical parameters in natural waters. J. Comput. Technol., 11: 39-45.
- 9 Maltsev V P, 2000. Scanning flow cytometry for individual particle analysis, Rev. Sci. Instrum. 71: 243-255.
- 10 Aristipini P, L Fiorani, I Menicucci & A Palucci, 2005. Spettrofluorimetro laser portatile per l'analisi in situ dei liquidi non opachi (Italian Patent Number: RM2005A000269)
- 11 Maltsev V P & A V Chernyshev, 1997. Method and device for determination of parameters of individual microparticles (United States Patent Number: 5650847)
- 12 Fiorani L, 1996. Une première mesure lidar combinée d'ozone et de vent, à partir d'une instrumentation et d'une méthodologie coup par coup (Swiss Federal Institute of Technology, Lausanne)
- 13 Barnaba F, L Fiorani, A Palucci & P Tarasov, 2006. First characterization of marine particles by laser scanning flow cytometry. J. Quant. Spectrosc. Ra., 102 : 11-17.
- 14 Semyanov K A, P A Tarasov, A E Zharinov, A V Chernyshev, A G Hoekstra & V P Maltsev, 2004. Single-particle sizing from light scattering by spectral decomposition. Applied Optics, 43, 5110-5115.
- 15 Guenther R, 1990. Modern Optics (John Wiley & Sons, New York)