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Second-order functions are the simplest correlations between flow cytometric light scatter and bacterial diameter

O. Julià^a, J. Comas^b, J. Vives-Rego^{c,*}

^aDepartament d'Estadística, Facultat de Matemàtiques, Universitat de Barcelona, Gran Via, 585, 08007 Barcelona, Spain

^bServeis Científic-Tècnics, Universitat de Barcelona; c/Lluís Solè Sabarís, 1-3, Barcelona, Spain

^cDepartament de Microbiologia, Universitat de Barcelona, Av. Diagonal, 645, 08028 Barcelona, Spain

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Abstract

Second-order mathematical relationships between bacterial cell diameter determined by electric particle analyser and flow cytometric forward light scatter in axenic cultures are obtained and discussed. Since it is technically impossible today to obtain both measurements for each individual cell, standard regression techniques cannot be applied. To overcome this limitation, we assume that these two parameters are related by a monotone increasing function that enables their mathematical relationships to be studied. Our conclusion is that forward light scatter data cannot be linearly transformed into bacterial size values by an accurate and universal function. However, second-order relationships seem to be the simplest satisfactory relationships between cell diameter and forward light scatter in eubacteria. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Flow cytometry; Particle size analyser; Distribution functions; Second-order adjustments; *Staphylococcus aureus*; *Micrococcus luteus*; *Salmonella typhimurium*; *Pseudomonas aeruginosa*; *Escherichia coli*

1. Introduction

Rapid methods of counting bacteria and measuring their size are required in medical, food and environmental microbiology. Flow cytometry has become an important tool that combines direct and rapid assays to determine numbers, cell-size distribution and additional biochemical analysis of individual cells (Shapiro, 1995; Robinson, 1999). Flow cytometric cell sizing is estimated from the intensity of forward light scatter, which is used in

preference to 90° scatter because of its high signal intensity and its insensitivity to subcellular structure. Forward scatter is assumed to be proportional to bacterial size (Heldal et al., 1994; Christensen et al., 1995; Button et al., 1996), although it is generally accepted that forward light scatter is also affected by the cell structure or chemical composition. Last years, the Rayleigh-Gans theory has been applied to predict biomasse in dry weight terms from forward scatter values (Koch et al., 1996; Robertson et al., 1996). However, no universal correlation between bacterial size and scatter has been described.

Sizing microbial cells during flow cytometric analysis is important in basic and applied studies of heterogeneous populations, and also in axenic cul-

*Corresponding author. Tel.: +34-93-402-1485; fax: 34-93-411-0592.

E-mail address: jvives@porthos.bio.ub.es (J. Vives-Rego)

tures (Davey and Kell, 1996). Studies of the relationship between scatter values and cell size should seek the simplest possible mathematical function according to the parsimonious principle (McCullagh and Nelder, 1989). The parsimonious principle states that considering all possible satisfactory models, the simplest is the best. In our opinion, there are two models in this context: first and second-order relationships. The aim of this paper is to show that the simplest and most satisfactory mathematical relationship between forward light scatter and bacterial diameter in axenic cultures is a second-order function.

2. Materials and methods.

2.1. Bacterial strains and culture conditions.

Experiments were performed with the following strains: *Escherichia coli* strain 536 (López-Amorós et al., 1994), *Staphylococcus aureus* ATCC 12600, *Micrococcus luteus*, isolated in our Department from a meat elaborate, *Bacillus polymixa* CECT 155 derived from ATCC 842, *Enterococcus faecalis* CECT 481 derived from ATCC 19433, *Salmonella typhimurium* ATCC 14028 and *Pseudomonas aeruginosa* CECT 110 derived from ATCC 10145. Cells were grown overnight in Luria Broth medium by incubation at 30°C and shaking at 300 r.p.m.

2.2. Flow cytometric analysis.

A Coulter Epics Elite flow cytometer equipped with an air-cooled 488 nm argon-ion laser at 15 mW power. Fluorescent beads (1 µ Fluoresbrite carboxylate microspheres, Polysciences Inc., Warrington PA, and 4 µm latex fluorosphere beads, Molecular Probes, Eugene, Oregon) were used as an internal standard for scatter and fluorescence. The forward scatter detector in the Elite flow cytometer is a photodiode that collects light between 1.5 and 19° from the laser axis, being able to discriminate particles >0.5 µm in diameter. The side scatter detector is situated at a 90° angle from the laser axis. Due to the design of the closed flow chamber used, light for both side scatter and fluorescence is collected in an angle wider than 90°, using a combina-

tion of mirror and lens in order to improve efficiency. Data were analysed with Elitesoft version 4.1 (Coulter Corporation) and WinMDI version 2.5 software (Joseph Trotter, 1996).

2.3. Cell size determinations

Cell sizes were determined with an electronic particle analyser, Multisizer II (Coulter Corporation), with an aperture tube of 30 µm in diameter and processing 100 µl of the cell suspension in 0.9% NaCl previously filtered through 0.2 µm. Three types of size measurement were obtained after the transformation of the electric pulses generated by the counter: diameter, volume and revolution surface. Data were analysed by AccuComp software version 1.15 (Coulter Corporation).

2.4. Theory

Our goal is to find a relationship, if it exists, between the measurements yielded by the scatter and the Multisizer II. Due to the fact that there is no technique available for obtaining both measurements in the same cell, standard regression techniques cannot be used. Thus, in this paper we use a different technique to calculate the relationship between the two parameters which is described next. Given a homogeneous population of microorganisms, let $F_d(x) = P(d \leq x)$ be the distribution function of the diameter d , that is $F_d(x)$ is the probability (P) that a micro-organism selected at random has a diameter less than or equal to x . In the same way, let $F_s(x) = P(l \leq x)$ be the distribution function of the light refraction l measured by the forward scatter. Assume that both measurements, diameter and light refraction, are related through a monotone increasing function $g(d) = l$, then:

$$\begin{aligned} F_d(x) &= P(d \leq x) = P(g(d) \leq g(x)) = P(l \leq g(x)) \\ &= F_s(g(x)) = (F_s \circ g)(x), \end{aligned}$$

when \circ means the composition of the functions. If F_s has an inverse, say F_s^{-1} , (and it is always possible when F_s is continuous and strictly increasing), g can be expressed as:

$$g = (F_s^{-1} \circ F_d) \quad (1)$$

That is, we can propose a calculation for g if good calculations for F_s , and F_d are available. For a sample of n micro-organisms, the empirical distribution function of the light scatter is:

$$\tilde{F}_s(x) = \frac{1}{n} \sum_{i=1}^m n_i I_{\{l_i \leq x\}}$$

where $l_1 \dots l_m$ are the different light scatter values, where I is the indicator function and n_i the number of micro-organisms having a light scatter l_i . Its plot is a step function since it remains constant between two consecutive values l_i, l_{i+1} and so is not suitable for our purposes. A simple modification on \tilde{F}_s , however, will smooth this function:

$$\bar{F}_s(t) = \frac{1}{n} \sum_{j=1}^{i-1} n_j + \frac{1}{n} \left(t - \frac{l_{i-1} + l_i}{2} \right) \cdot \frac{2n_i}{l_{i+1} - l_{i-1}} \quad \forall t \in \left[\frac{l_i + l_{i-1}}{2}, \frac{l_i + l_{i+1}}{2} \right]$$

and

$$\forall i = 1, 2, \dots, m \text{ (where } l_0 = l_1, l_{m+1} = l_m)$$

Note that \bar{F}_s is a linear interpolation between the mid-point of each constant part of \tilde{F}_s . The same procedure is applied to obtain \bar{F}_d . A calculation of g can be obtained using \bar{F}_s , \bar{F}_d and Eq. (1): $\bar{g} = \bar{F}_s^{-1} \circ \bar{F}_d$ where \bar{g} is the estimation of g .

Files generated by the particle size analyzer (Multisizer) were exported in an ASCII (tab delimited) format. Listmode files generated by the flow cytometer were opened with the WinMDI software ver. 2.7 (Josep Trotter, 1998). Forward Scatter (FSC) was saved as a single parameter in an FCS ASCII format. The files thus generated were opened and formatted to a single column using a self-made Microsoft Word macro. A C-language program which computes \bar{g} starting from Multisizer II and forward scatter data has been developed by Olga Julia. This program is freely available to non-profit institutions upon request.

3. Results and discussion

The first attempt to obtain a correlation between cell diameter, volume or revolution surface and the forward light scatter using lineal correlations and

logarithmic transformations was completely unsatisfactory. The second attempt, which looked for second-order relationships between forward scatter and cell volume and revolution surface, was also unsatisfactory. The simplest and most satisfactory relationship was obtained for second-order adjustments between forward light scatter and cell diameter values.

We measured \bar{g} , calculation of the g function, using five samples of 10 000 organisms for each measuring method (scatter and multisizer). The observed behaviour in the range containing the central 90% of the population distribution, suggests fitting g to a second-order polynomial, $y = ax^2 + bx + c$, where y is the diameter calculated from the forward scatter values, x is the forward scatter value given by the flow cytometer, a , b and c are the coefficients of the second order equation, being c the intercept. Such a type of adjustment is specially satisfactory in the cases of *Pseudomonas aeruginosa* and *E. coli*. Scatter values given by the flow cytometer ranged from 1 to 1024, and the diameter measured in microns (μ) by the Multisizer II, ranged from 0.7 to 3 μ . Scatter values were scaled by 10^{-3} in order to approach both ranks. Least-square fitting yields the following coefficients:

<i>P. aeruginosa</i>	$a = 5.05(x^2)$	$b = -3.62(x)$	$c = 1.42$
<i>E. coli</i>	$a = 8.54(x^2)$	$b = -6.71(x)$	$c = 2.08$
<i>Salmonella</i>	$a = 6.17(x^2)$	$b = -4.09(x)$	$c = 1.41$
<i>S. aureus</i>	$a = 9.41(x^2)$	$b = -7.23(x)$	$c = 2.20$
<i>M. luteus</i>	$a = 16.58(x^2)$	$b = -10.94(x)$	$c = 3.04$

For example, in the case of *P. aeruginosa*, when we have a forward light scatter value of 500, considering that the coefficients for this strain are: $a = 5.05$, $b = -3.62$ and $c = 1.42$, the calculated diameter (y) is 0.87 μ .

In order to verify the accuracy of g calculation, we transformed the forward scatter data using \bar{g} function; then a plot of the Multisizer II data quantile vs. transformed data quantile was obtained. The proximity of the plot to the straight line shows the similarity of the distributions (Fig. 1). All the computations used S-PLUS software (Statistical Sciences, Inc. Seattle, WA 98109 USA).

The apparent visual coincidence of the solid line with the open-circle line in the graphs of the left

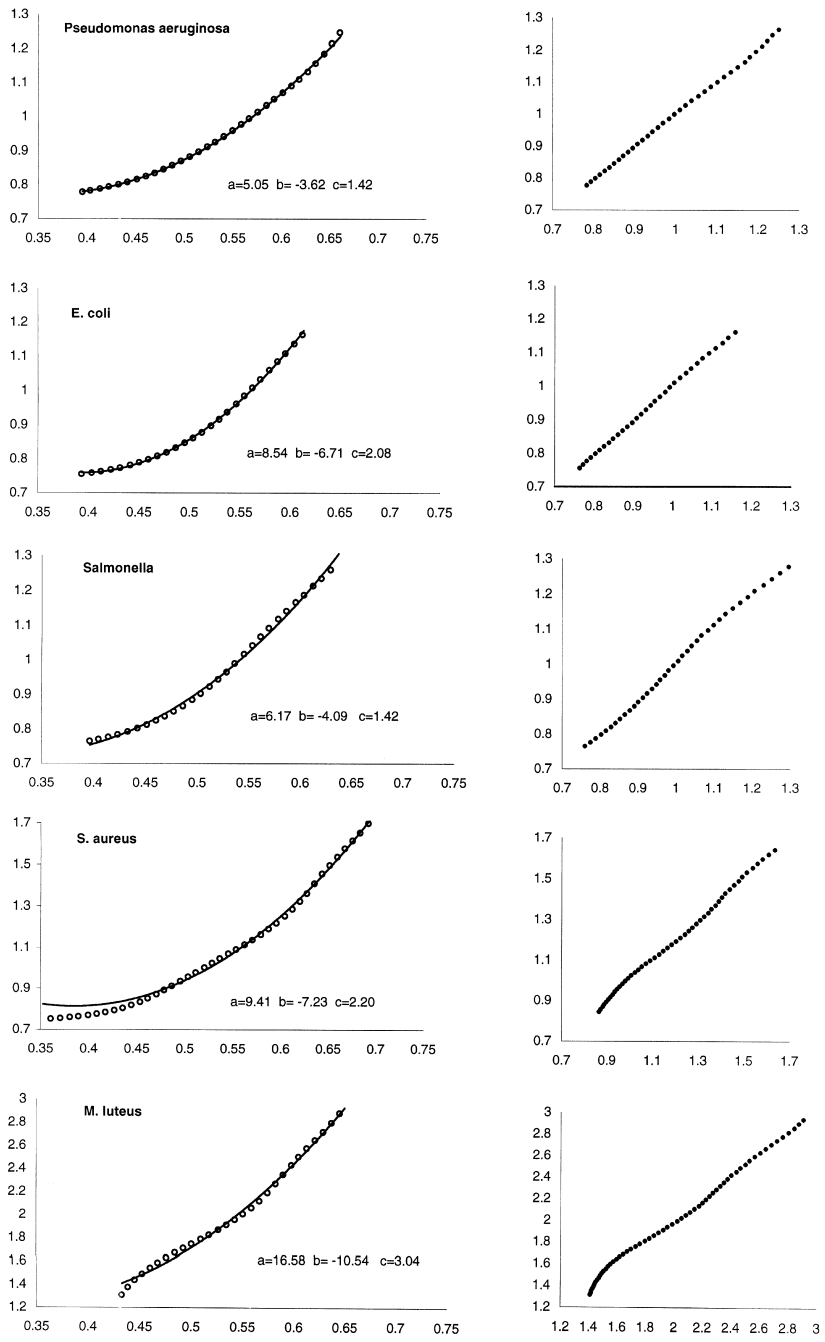


Fig. 1. The graph in the left column show the relationship between the forward light scatter and diameter measurements for individually species. In the Y axis, the diameter of the cells in microns as obtained by the particle analyser (Multisizer II) is represented. In the X axis, scatter values obtained by the flow cytometer scaled by 10^{-3} , are represented. The open-circles line represents the scatter values transformed by \bar{g} . The solid line shown the second-order ($y = ax^2 + bx + c$) adjustment of the scatter values transformed by \bar{g} ; a , b , c values shown inside the graph of each species and corresponds to the coefficients of the second-order function. The graphs in the right column shown the verification of the \bar{g} estimation: multisizer data quantile are plotted against \bar{g} function-transformed scatter data quantile.

column in Fig. 1 clearly show that the quality of the adjustment was in decreasing order: *P. aeruginosa*, *E. coli*, *Salmonella*, *S. aureus* and *M. luteus*. The quality of the adjustments for each bacteria is corroborated in the right column of the graphs, in which the better the linearity, the better the adjustment. The obtained adjustments for the different bacteria, which in fact there are not so different in size, differ from each other. This is especially so in the case of the two cocci, which indicates strongly that no universal correlation for any kind of bacteria.

Although scatter can not be converted universally into cell size measurement, the differences in forward scatter represent proportional differences in cell size. The second-order correlation obtained is only well-adjusted when the central 90% data of the obtained distributions. When 100% of the distribution data are used to obtain the correlations, considerable distortions are observed, which suggests that the bacteria on both sides of the distribution show anomalous size and scatter values.

Lately, the Rayleigh-Gans theory has been applied to predict biomass in dry weight terms from forward scatter values (Koch et al., 1996; Robertson et al., 1996), and authors state that this method is only suitable for small bacteria. In fact, dry weight and bacterial size is not always correlated in laboratory cultures or in natural bacterial aquatic populations. Since bacterial size is a data of basic and applied interest, our second-order relationship between bacterial diameter and forward scatter provides a rapid and useful method to assess bacterial size in axenic cultures.

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