A Rapid Method for the Determination of Absolute Particle Sizes in a Flow Cytometer

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Size determination of a sufficiently large number of cells is a problem which very often arises when cellular parameters should be normalized. This task is most simply performed under a light microscope by measuring the cells one by one and calculating their mean diameter. Improving this method leads to a determination of orthogonally orientated diameters. In any case, this one-dimensional determination of a length runs the risk of a subjective selection, especially when irregularly shaped cells are investigated; it is not only unprecise but also time-consuming.
An automatic image-processing of light microscopical pictures of cells seems to be an attractive alternative. Within a short time it analyses many cells by integrating their projected areas. This two-dimensional determination of an area is more precise than a simple measurement of a length. It requires, however, a highly sophisticated instrumentation for a relatively simple problem and still deals with a too small number of cells.
The most precise determination of a cell-size distribution can be expected by registering the signal of a three-dimensional size (particle volume). This is best performed in a flow cytometer with an integrated volume measurement according to the Coulter-principle (Coulter, 1956). This electrical measurement is very fast and is more precise than the forward-scatter method which is used alternativly in flow cytometry.
The mean size of particles which has been determined by the Coulter-principle is mostly given by arbitrary units. We, therefore, present here a simple and fast calibration method which allows the transformation of arbitrary to absolute values. In addition we compared these data with one- and two-dimensional measurements under the microscope and an image-processing-system, respectively.

Material and Methods

Latex-beads

All three mentioned systems for measuring particle sizes were calibrated with latex-beads as listed in table 1. For flow cytometry measurements the latex-beads were diluted in isotonic salt solution Ultra-Count (Becton Dickinson, Heidelberg, FRG), under the microscope undiluted suspensions were used.

Table 1: Latex-beads with different diameters which were used for calibrations.

Manufacturer	indicated diameter / µm
Flow Cytometry Standard Corporation (North Carolina, USA)	7.50*
	8.40**
Polyscience (Warrington, USA)	15.80
	25.70
Coulter Calibration Standards (Luton, Beds., UK)	5.96
	9.70
	14.50
	19.20

labelled with: * Fluoresceinisothiocyanat
** Phycoerythrin

Cell Lines and Cell Culture

Three cell lines which differed in size and morphology were used as cell suspensions to compare the methods for size determination. All cell lines were maintained at 37°C under an atmosphere of 8 % CO_2 in air at pH 7.4.
L1210 cells, a lymphocytic mouse leukemia cell line (Hutchison et al., 1966), grew as suspension in RPMI-medium (Biochrom KG, Berlin, FRG) with $NaHCO_3$ and 15 % fetal calf serum (Boehringer Mannheim, FRG). Prior to use, 100 µl mercaptoethanol and 2 ml sodiumpyruvate were added to 100 ml medium. The average cell concentration was about 2×10^5 cells/ml. L1210 cells are very small and of uniform spheroidal shape (fig. 1a,b).
BICR/M1R_k-cells are fibroblastoid growing cells (fig. 1c) of a transplantable mammary tumor of the Marshall rat (Rajewsky & Grüneisen, 1972). These cells were kept in DMEM (Biochrom) supplemented with 10 % newborn calf serum (Boehringer Mannheim) as monolayer cultures. Separated in a single cell suspension after treatment with 0.25 % trypsin and 1 mM EDTA in Ca^{++}- and Mg^{++}-free phosphate buffered salt solution, these cells are relatively big and their shape deviates considerably from a spheroid (fig. 1d).
F9-cells are embryonic carcinoma cells which originate from the testicular teratocarcinoma of the mouse strain 129 (Bernstine et al., 1973). These cells grow as densely packed colonies

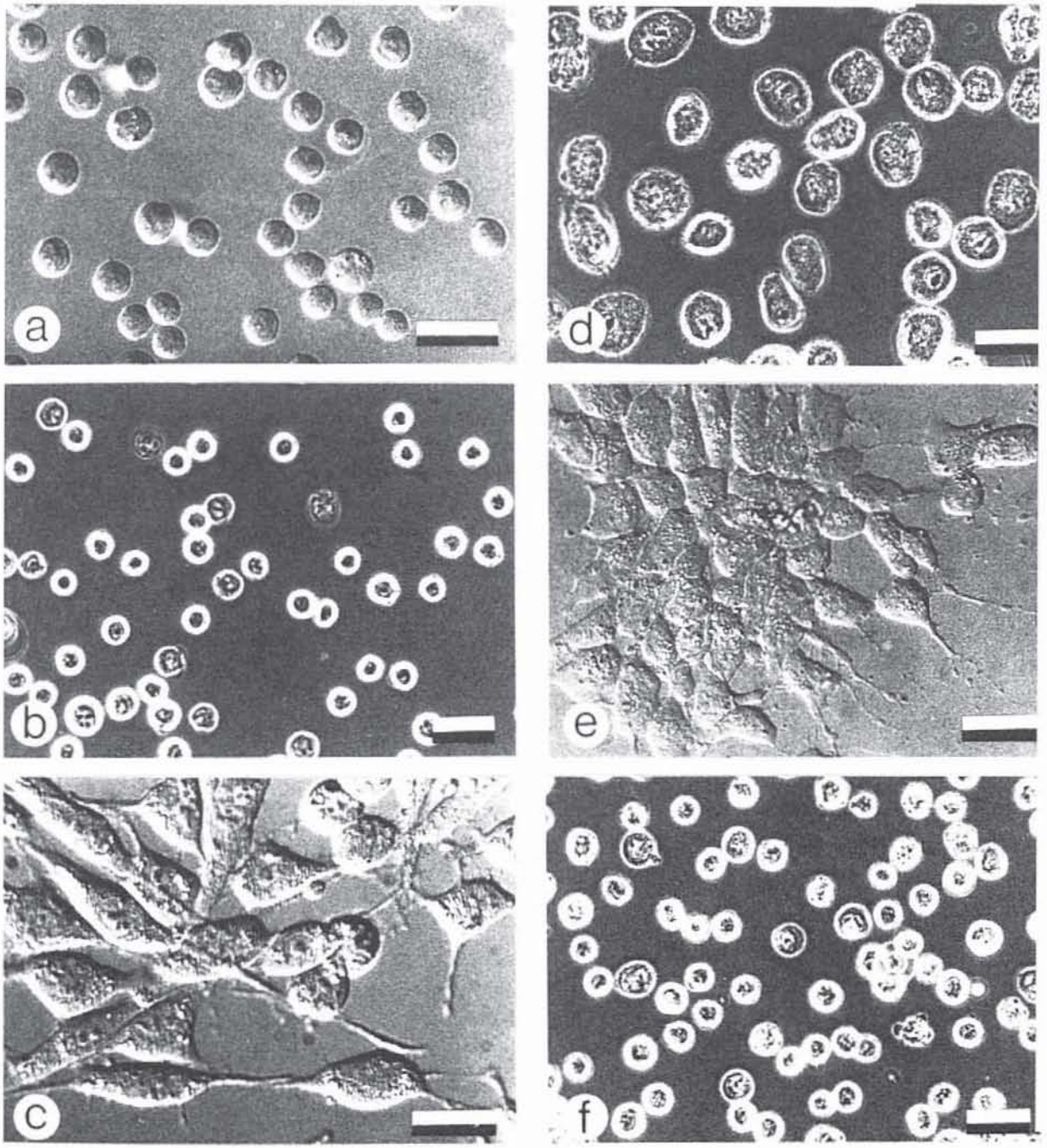

Fig. 1: Different cell lines grown in suspension (a) or as monolayer culture (c, e) were used for size determination. Cells growing as monolayers were separated with trypsin to single cell suspensions (d, f).
a,b: L1210-cells; c,d: BICR/M1R$_k$-cells; e,f: F9-cells
a,c,e: Differential Interference Contrast; b,d,f: Phase Contrast
Bar: 30 μm

(fig. 1e) on gelatine coated (0.1 %) petri dishes in DMEM with 10 % fetal calf serum. When separated and suspended these cells are relatively small and exhibit a rather uniform spheroidal shape (fig. 1f).

Determination of Size

For the microscopical size determination pictures of particles and cells were taken with a Zeiss Axiophot (Zeiss, Oberkochen, FRG; phase contrast, water-immersion objective 40×). Their diameters were measured by projecting the film together with a scale. Samples of 200 particles or cells are given as mean ± s.d. (standard deviation).
For the size determination with an image-processing-system (Kontron, München, FRG) particles, cells, and a scale were recorded through a Zeiss IM 35 inverted microscope (phase contrast, water-immersion objective 40×) with a monochrome chalnikon tube video camera. Data-analysis was performed with an image-processing program (U. Reichl and E.-D. Gilles, Institut für Systemdynamik und Regelungstechnik, Universität Stuttgart) which determined the area of individual particles or cells and calculated the theoretical diameters of circles with identical areas. Again, the mean ± s.d. is given from samples of 200 particles or cells. The flow cytometer FACS-Analyzer (Becton Dickinson, Mountainview, USA) was used for sizing the cells in capillaries of different diameters. Every capillary was calibrated at its recommended optimum current with matching latex-beads. The amplification was set logarithmic; variable gain and threshold were set at minimum. Isotonic salt solution Ultra-Count was used as sheath fluid. Samples of 10,000 particles or cells were analysed and their diameter is presented as mean ± c.v. (coefficient of variation) to characterize the size distribution.

Theory of the Coulter-principle

The Coulter-principle (Coulter, 1956) sizes a particle by registering a change in ohmic resistance which occurs when a nonconducting particle passes with the laminar flow of an electrolyte through a capillary. Because of its small diameter (orifice max. 150 μm in a FACS-Analyzer) this capillary represents the highest ohmic resistance in the tubing of a flow cytometer. Every change of ohmic resistance by nonconducting cells, therefore, is best registered at the capillary (fig. 2).
Electrodes which are located at both ends of the capillary are connected to a current source and maintain a constant current of some milliamperes across the capillary. Reducing the profile within the orifice by the passage of a nonconducting particle, therefore, increases the potential difference across the capillary. This voltage signal is proportional to the particle size and its time course during the passage of a particle through a long capillary is trapezoid.

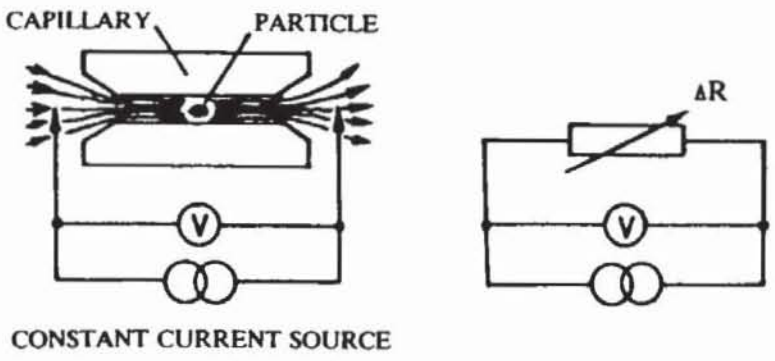

Fig. 2: Schematic drawing and circuit diagram of the Coulter-principle.

The change of the ohmic resistance within a given capillary depends on several factors:

- particle size
- particle shape
- orientation of the particle
- deformation of the particle
- electrical conductivity of the electrolyte
- electrical conductivity of the particle
- homogeneity of the electric field

This listing indicates the difficulties for a correlation of resistance changes with absolute particle sizes. Precise measurements, theoretical considerations, and mathematical derivations which include these parameters have been made by Kachel (1979, 1982) who also reviews the latest literature.
Shape and size of mammalian cells depend on many physiological parameters and sizing of such populations, therefore, is always limited by their natural variance which greatly reduces the requirements for the accuracy of size determinations. Our presented simple method for calibrating the Coulter-principle allows a quick determination of absolute cell sizes with an accuracy better than their natural variance. This derivation is written in electrical terms. In contrast, the measured and calculated values have arbitrary units which formally correspond to the electrical units.
With solid, spheroid, and nonconducting particles the change of resistance within the capillary is only dependent on particle size, whereas the resistance of the capillary depends

on the resistivity of the electrolyte(ρ; $\Omega \cdot \mu m$), on the length of the electrolyte filled capillary (l, l_C; μm), and on the area of the capillary apertur (orifice) (A ,A_C; μm^2):

$$R = \frac{\rho * l}{A} \tag{1}$$

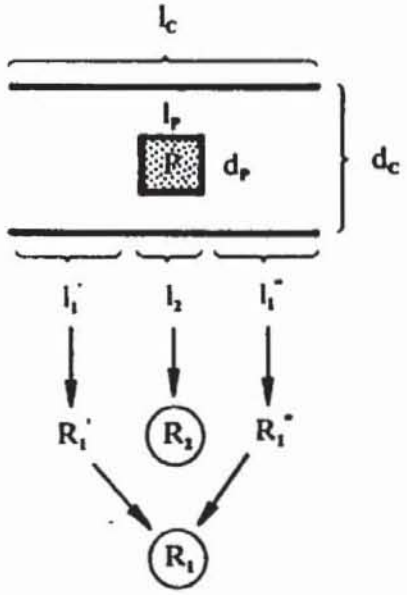

Fig. 3: Subdivision of a capillary containing a cylindrical particle ($l_P = d_P$) into different regions of electrical resistance.

The capillary can be subdivided in different regions of resistance as is shown in fig. 3. Without particles the resistance of the capillary is

$$R_C = \frac{\rho * l_C}{A_C} \tag{2}$$

As long as the particle size is small compared with the diameter of the capillary the shape of the particle can be neglected (Kubitschek, 1960). Under these conditions, any change in the capillary resistance ΔR which is caused by a particle with the area A_P and the length l_P can be used for the determination of its size as is shown by the following equations:

$$\Delta R = R_1 + R_2 - R_C \tag{3}$$

$$\Delta R = \frac{\rho * l_P}{A_C * (A_C / A_P - 1)} \tag{4}$$

Equation (4) has been derived for any cylindrically shaped particle. The determination of unknown cell sizes must be preceded by a calibration with well defined latex-beads. These beads are spherical and must not necessarily fit the assumption for cylindrically shaped particles. However, by reducing the length l_P of the particle to its diameter d_P, this equation may also be used for spherical particles within the limitations of the required resolution.
With $l_P = d_P$ and $A = \pi \cdot (d/2)^2$
equation (4) becomes

$$\Delta R = \frac{\rho * 4 * d_P}{\pi * d_C^2 * ((d_C / d_P)^2 - 1)} \tag{5}$$

The determination of absolute cell sizes requires the solution of equation (13) for d_P. This leads to the cubic equation

$$4 * \rho * d_P^3 + \Delta R * \pi * d_C^2 * d_P^2 - \Delta R * \pi * d_C^2 * d_C^2 = 0 \tag{6}$$

which can be solved by an approximation

$$d_P = d_C * \sqrt[3]{\frac{\pi * \Delta R * d_C}{4 * \rho}} - \underbrace{\frac{1}{3} * \frac{\pi * \Delta R * d_C}{4 * \rho}}_{\textit{extension term}} \tag{7}$$

where the extension term is negligible.
We thus end with an equation which allows the determination of absolute particle sizes. The constant factors are pooled to the "capillary constant" K_C which is obtained for every capillary by calibrating with particles of defined sizes.

$$d_P = K_C * \sqrt[3]{\Delta R} \tag{8}$$

with

$$K_C = \sqrt[3]{\frac{\pi * d_C^4}{4 * \rho}} \tag{9}$$

Since

$$d_P^3 \sim V_P \tag{10}$$

equation (8) corresponds to the relation between particle volume V_P and measured resistance change ΔR as has been found by Coulter (1956) by a different mathematical derivation:

$$V_P \sim \Delta R \tag{11}$$

An estimation of the influence of particle shape on ΔR and, therefore, on the calculated absolute particle size d_P, is obtained when two differently shaped cylindrical particles with the same volume are compared: particle 1 with $l_{P1} = d_{P1}$ and particle 2 with $l_{P2} = 8 \cdot d_{P2}$, thus

$$d_{P1} = d_{P2} * \sqrt[3]{8} \tag{12}$$

With the relation between ΔR and the particle volume we can calculate the influence of the particle shape on ΔR.

$$V_{P2} = V_{P1} \tag{13}$$

$$\frac{\Delta R_2 * A_C * (A_C - \pi * (d_{P1}/2)^2 * 1/\sqrt[3]{8^2})}{\rho} - \frac{\Delta R_1 * A_C * (A_C - \pi * (d_{P1}/2)^2)}{\rho} \qquad (14)$$

$$\Delta R_2 = \Delta R_1 * \frac{A_C - \pi * (d_{P1}/2)^2}{\frac{A_C - \pi * (d_{P1}/2)^2 * 1/\sqrt[3]{8^2}}{\textit{shape factor}}} \qquad (15)$$

Equation (15) shows that the changes in capillary resistance ΔR for particles of the same volume but different shapes are not identical. ΔR depends not only on the particle volume but also on the particle shape by a factor which contains the diameter of the particle and the capillary. This relation is shown in fig. 4 for all four capillaries.
As has been mentioned above, this derivation is written in electrical terms and with the assumption of a constant electrolyte resistivity ρ. Since the electrical field in an electrolyte filled capillary is inhomogenous (Kachel, 1979), every capillary is characterized by an individual resistivity ρ_C. When determined at the recommended optimum current, ρ_C is constant for a given electrolyte filled capillary. The capillary constant K_C can be obtained, therefore, by calibrating with different particles.
Solving equation (5) for ρ_C leads to:

$$\rho_C = \frac{\Delta R * \pi * d_C^2 * ((d_C/d_P)^2 - 1)}{4 * \rho} \qquad (16)$$

Since in the flow cytometer values for ΔR are measured in arbitrary units ranging from 1 to 1000, ρ_C is also given in arbitrary units. With this procedure it is possible to determine absolute sizes of unknown particles (or cells) by measuring their ΔR. It is, therefore, unnecessary to know the absolute electrical values for ΔR and ρ_C when using equation (8).

Results

Sizing by Flow Cytometry

Four capillaries with diameters of 50 μm, 75 μm, 100 μm and 150 μm were characterized by their capillary constant K_C. For this purpose at least five probes of latex-beads with

different sizes were analysed. With the measured ΔR the resistivity ρ_C was calculated according to equation (24) and the capillary constant K_C according to equation (21) (table 2). From these data a calibration curve for every capillary was computed by inserting ΔR and K_C in equation (20) (fig. 5). This can be used to investigate the influence of capillary diameters on the determination of particle sizes. In fig. 6 the particle size as indicated by the manufacturer is compared with the measured values in four capillaries. Our measurements reveal that the orifice has no influence on sizing the particles, at least when their diameter does not exeed 35 % of the orifice. Every capillary shows an excellent linear correlation between indicated and measured values, closely matching the theoretically expected diagonal as can be seen from the regression analysis in fig. 6 and 7c. For bigger particles a deviation from the fitted curve can be seen. The accuracy of all measurements, however, is evident from fig. 7c where the particle sizes measured with different capillaries are combined and given as mean ± s.d.; in every case the standard deviation is smaller than the dots in the diagram.

The influence of the particle shape on ΔR can be estimated from fig. 4. The absolute particle size must be calculated with equations (23) and (20). For particles with a diameter of 35 % of the orifice (particle 14.5 μm, orifice 50 μm) this shape factor is about 0.935, resulting in a reduced value of about 2.2 % for the absolute particle size. With increasing ratio of orifice to particle diameter the influence of the particle shape on the size determination is considerably reduced, e.g. to 0.3 % (shape factor = 0.995) for the 14.5 μm particle in an orifice of 150 μm. Assuming a deformation of the mammalian cells when passing the capillary, this shape factor is about 0.96 for BICR/M1R$_k$-cells which have been measured in the 75 μm orifice. Thus, the deviation from the measured value of 17.9 μm is less than 0.3 μm for this big cells and is even less for the smaller L1210-cells (11.9 μm) and F9-cells (11.3 μm).

Sizing by Light Microscopy and Image-processing

The microscopical determination of particles resulted in a high correlation between indicated and measured values (fig. 7a), the fitted curve, however, deviates considerably from the theoretically expected diagonal. Obviously, a systematical error in determining the particle size cannot be avoided with this subjective one-dimensional measurement.
In contrast, two-dimensional measurements with an image-processing system revealed a high correlation between indicated and measured values (fig. 7b) with almost no deviation from the theoretically expected diagonal.
Interestingly, for the 14.5 μm particles a deviation to smaller measured values can be seen with all three methods. Comparing the flow cytometry measurements with the results obtained with the two other methods, it is obvious that the one-dimensional determination is less accurate (fig. 8a), whereas the image-analysis method is as accurate as the flow cytometry measurement (fig. 8b).

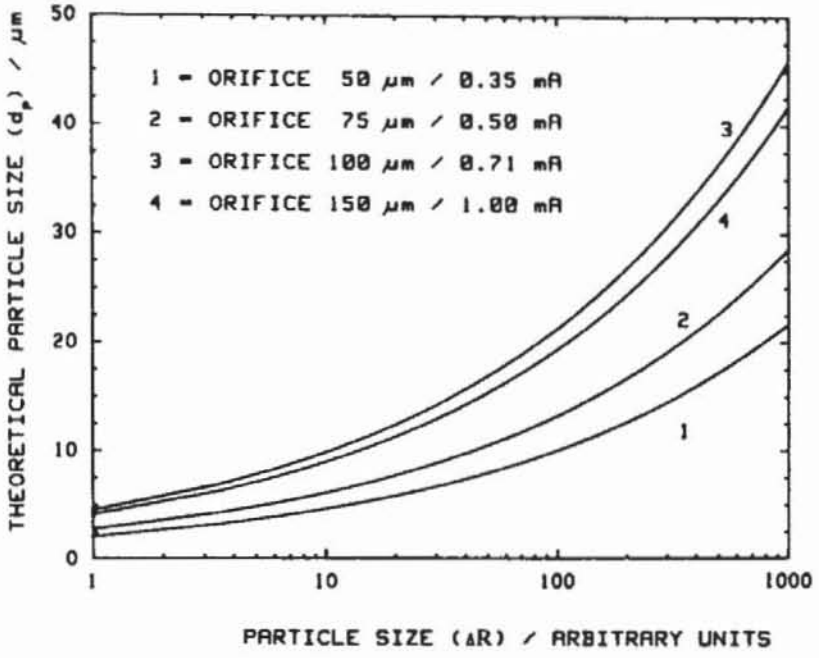

Fig. 4: Computed shape factors of equation (23) for different capillaries. The particle diameter d_{P1} describes a "spherical" particle ($l_{P1} = d_{P1}$), the shape factor is calculated for longish particles with a cylindrical shape ($l_{P2} = 8d_{P2}$) but with the same volume as particle 1.

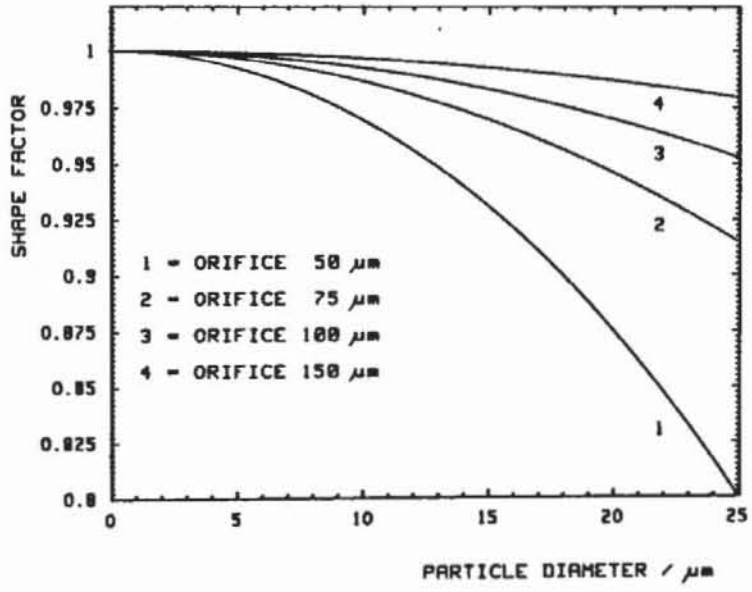

Fig. 5: Computed calibration curves (equation (20)) for different capillaries (50 µm, 75 µm, 100 µm, 150 µm) in the FACS-Analyzer.

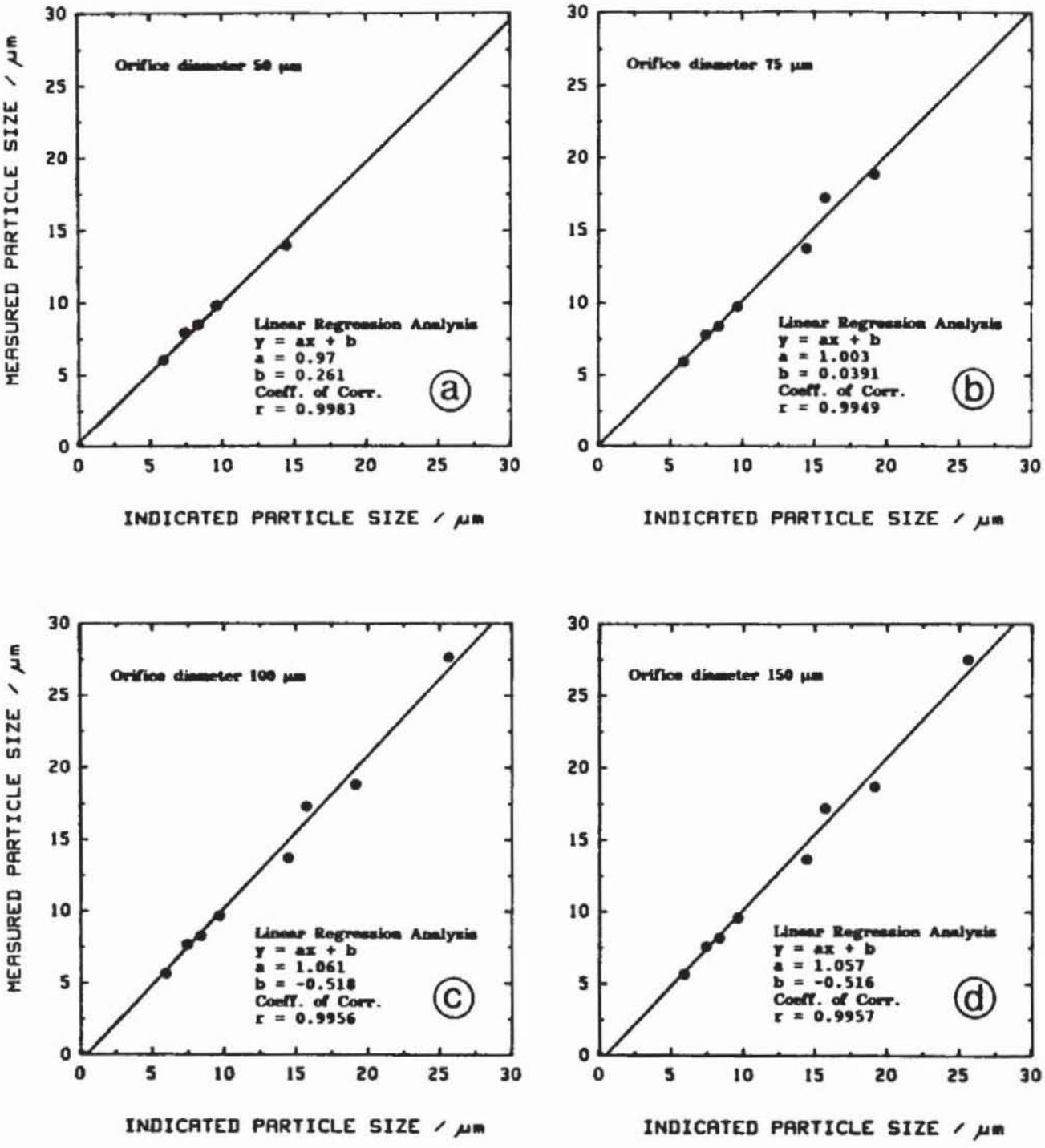

Fig. 6: Comparison of different capillaries for the size determination of particles. The diagrams show the relation between measured and indicated size of latex-beads.

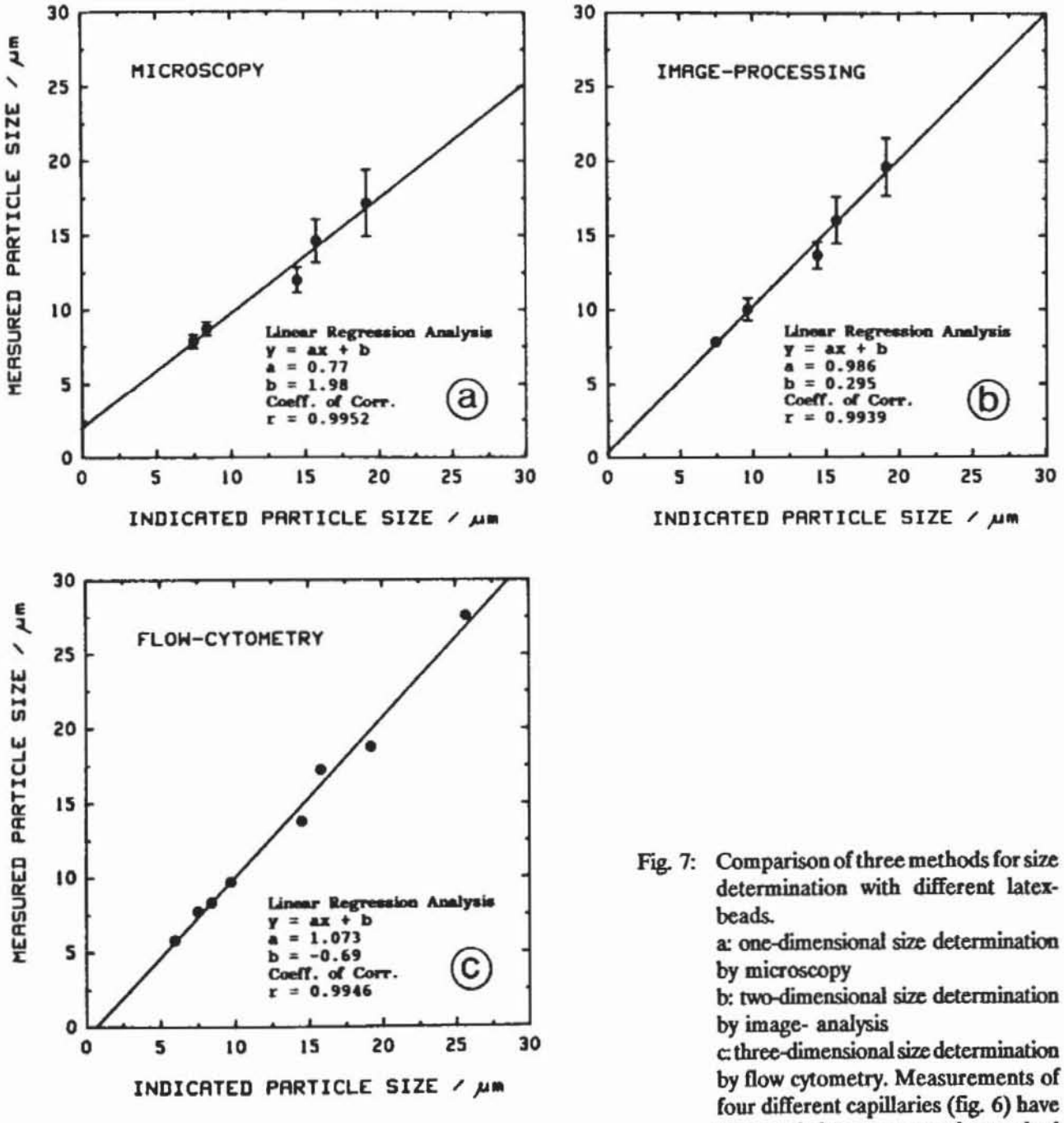

Fig. 7: Comparison of three methods for size determination with different latex-beads.
a: one-dimensional size determination by microscopy
b: two-dimensional size determination by image- analysis
c: three-dimensional size determination by flow cytometry. Measurements of four different capillaries (fig. 6) have been pooled. In every case the standard deviations of these mean values are smaller than the dot.

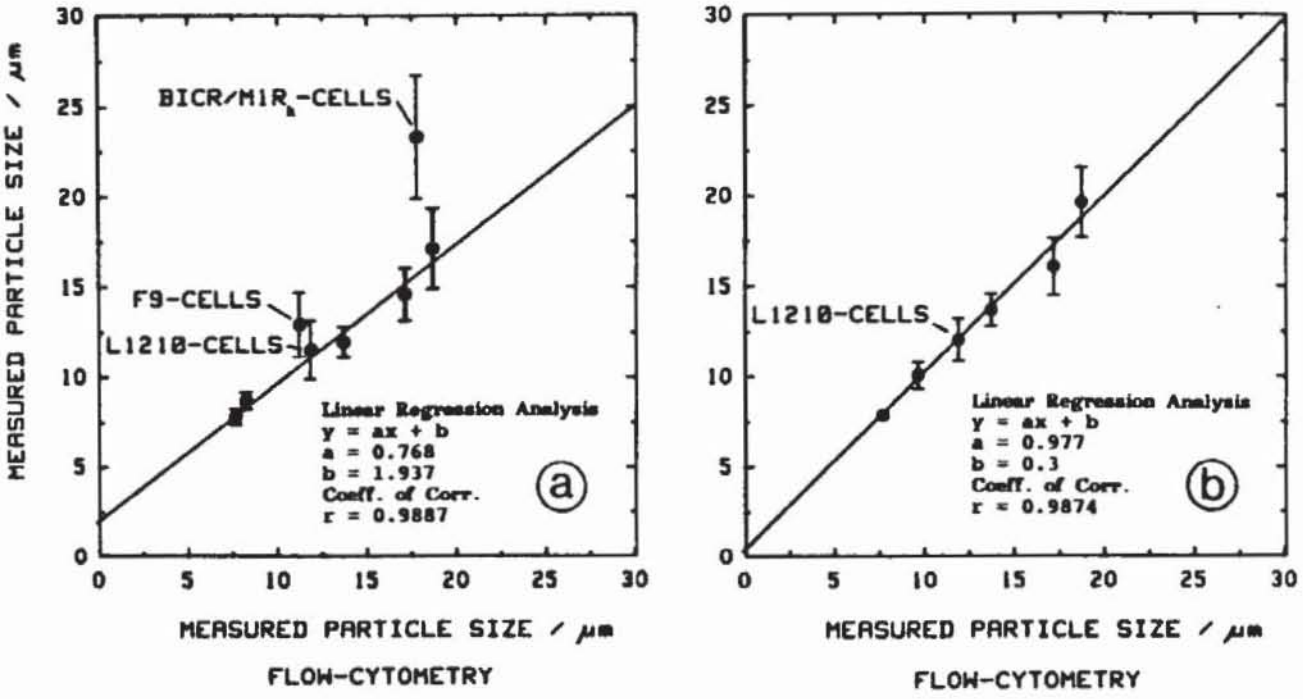

Fig. 8: Comparison of the size determination by microscopical measurements (a) and by image-analysis (b) with the size determination by flow cytometry of latex- beads and mammalian cells.

Conclusion

We have shown that a simple and quick calibration of particle sizes with the Coulter-principle and the application of a simplified mathematical equation allow an exact determination of particle sizes in flow cytometry. The data for the calibration curve originate from measured electrical parameters, however, no information about the electrolyte conductivity and the electrical current is necessary. The capillary resistance ΔR changes according to the size of a passing particle. The resistivity ρ_C of an electrolyte filled capillary, however, is constant for a given capillary. This leads to the definition of a capillary constant K_C which must be evaluated for every capillary at its recommended optimum current. This capillary constant is essential for the calculation of the calibration curve (fig. 5) which allows a determination of the absolute size of unknown particles.

An advantage of our method is the determination of the capillary constant K_C by a calibration with particles of different sizes. This averaging reduces the influence of falsely indicated particles as can be seen with the 14.5 μm latex-beads which always deviated to smaller values.

A comparison of the three methods demonstrates that the one-dimensional microscopical determination of particle sizes is the most inaccurate method. It is influenced by systematic errors as well as by the fact that an evaluated diameter of an irregularly shaped particle is

not characteristic for its three-dimensional size. This is obvious from fig. 8: with increasing cell size and irregularity of cell shape (L1210 → F9 → BICR/M1R$_k$) the microscopically measured cell sizes deviate to a greater extent from the cell size determined by flow cytometry. In addition, this method is time consuming and, therefore, unpracticable. The size determination with an image-processing system also requires a microscopical picture, but the two-dimensional area of an irregularly shaped particle represents a better value for its size. Because of an automatic or an interactive process, the time for the size determination is reduced. Difficulties occur when particle borders are not clearly detectable by the image-processing-system and when the cells spread rapidly.

The size determination with the Coulter-principle is the most accurate and fast alternative, because the signal of a three-dimensional size (particle volume) is registered with a high flow rate. If the particle shape deviates extremly from the assumed "spherical" shape ($l_P = d_P$), shape factors have to be considered, especially when the particle size is large compared with the orifice (fig. 4). The enormous deviation of the BICR/M1R$_k$-cells from the fitted straight line in fig. 8a, however, originates from the inaccurate microscopical measurement. If we assume that these cells can deviate from the spherical shape (l:d = 8:1) than we can determine the shape factor from fig. 4 and equation (23). The size of these cells (17.9 μm) corresponds to the 19.2 μm latex-beads for which the calculations have been made. With the orifice 75 μm a shape factor of 0.95 can be taken from fig. 4. The calculated absolute particle size (equation (20)) is 18.51 μm for the longish instead of 18.84 μm for the "spherical" particle with the same volume. This indicates a negligible error of 1.75 % in sizing deformable cells in the 75 μm orifice.

As the electric field in the capillary is inhomogenous, ΔR of big particles also depends on this parameter (Kachel, 1979). The comparison of the four capillaries, however, reveals (fig. 6, fig. 7) that the inhomogenous electric field has no effect on the calculated absolute particle size d_P within the investigated range of particle sizes. Even with an orifice to particle ratio of 3.4 (capillary 50 μm, particle 14.5 μm) the calculated absolute particle size d_P does not differ significantly from values determined with the bigger capillaries, where the ratio has increased to 10.3 (capillary 150 μm, particle 14.5 μm).

By calibrating with particles of different sizes, individual capillary constants K_C can be computed. Since the inhomogeneity of the electric field is integrated in the individual K_C, ΔR depends mainly on the particle volume. As we have shown, a considerable change in the shape of a particle is of minor influence. This method can, therefore, be used to determine sizes of deformable mammalian cells with sufficient accuracy.

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