BUGS IN THE BEAM

A Manual for Cytometry in Microbiology

Handouts for the Tutorial on Microbial Flow Cytometry

See also
Special issue free full-text issue of the Journal of Microbiological Methods

Volume 42/1, September 2000, Microbial Analysis at the Single Cell Level,
guest-edited by: L. Alberghina, D. Porro, H. Shapiro, F. Srienc, H. Steen.
See
http://www.elsevier.com/homepage/sah/mimet/speciss/1378.pdf

and
http://intl.highwire.org/
to receive freely available articles (published normally more than 6 month to a year ago)

The Program:
The aim of the tutorial is to give flow cytometry users the confidence and the technical background to tackle the measurement of bacteria. To achieve that there will be a theoretical part and presentations of practical applications, accompanied by protocols and reference literature

Gerhard Nebe-von Caron
Swiss Precision Diagnostic
Priory Bus. Park,
Bedford
Bedfordshire
GB - MK44 3UP
Tel.: +44-(0)1234-835474
FAX: +44-(0)1234-835002
E.mail: gerne@bigfoot.com

1 Background Information

The direct microscopical observation of "animalcules" by Leeuwenhoek in 1674 as described in his letters to the British Royal Society has been one of the key events of science of the last few centuries. It facilitated the understanding of the single cell nature of bacteria. The fact that one of these small organisms can give rise to an entire culture or colony has given microbiologists a single cell analysis system of outstanding detection sensitivity without the need for high tech equipment. The high amplification factor from 1 cell to 10^{10} cells and more, and the simple visual detection gave rise to a variety of microbiological tests based on cell growth.

The improvement in microscopic analysis of stressed and injured cells or the observations in extreme environmental conditions, in particular in connection with fluorescent probes, have highlighted the discrepancies between bacterial existence and their replication. The experience of replication in the form of raising children can give a 'macroscopic' insight in the stress and lifestyle changes caused by such process. To avoid similar distortions caused by post sampling growth, it appears that observations into natural microbial populations have to be based on direct optical detection methods on the single cell level.

1.1 Cytometry, bulk and single cell measurements

Because of the importance of microbiology to human health, methods have been developed to enumerate bacteria to identify them and to look at the impact of physical, chemical of biological interventions. Bulk measurements like changes in turbidity, conductivity or gas pressure of liquid media (Figure 1) have become popular for bacterial detection because of their ease of handling, their detection speed. Selective growth media can allow some degree of bacterial differentiation, but detailed differentiation is still achieved by cell isolation followed by either biochemical, immunological or genetic characterisation. Whilst immunological and genetic differentiation can also be applied directly to certain samples, preenrichment steps are usually applied to generate sufficient signal.

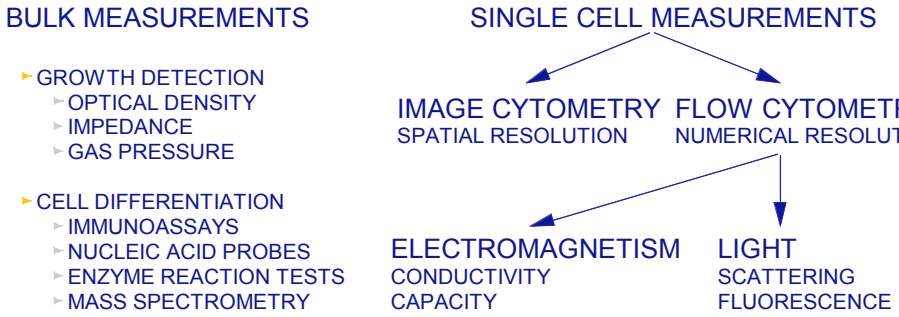


Figure 1: Cytometry as bulk or single cell measurements

Bulk measurements are usually easy to perform and less expensive. In most cases cell growth is required to generate enough signal. Direct single cell measurements on the other hand tend to be more complex. They do not require post sampling growth and can reflect the true heterogeneity of microbial populations.

The cornerstone of microbiology has been single cell analysis. Colonies derived from single cells have been examined by the plating techniques developed by Koch more than a century ago. The strength of this technique, the high amplification factor of $10^{9\text{-}12}$ is also its weakness, the dependence on growth. In the times of Pasteur and Koch as well as nowadays, this growth limitation can only be overcome by direct single cell measurements like image or flow cytometric methods, which also allow assessment of the true amount of sample heterogeneity. The power of the combination of image analysis and microscopy was already appreciated by Koch, who took pictures of his microscopic images. The spatial resolution of the microscope not only allows the characterisation of cell morphology, but also the position of bacteria within a sample matrix. This can give information about its development of biofilms or potential symbiotic interactions. Unfortunately the high amount of data processing in computerised image analysis limits the sample throughput and the analysis of high cell numbers, which are better achieved by the measurement of cell suspensions by flow cytometry (FCM). Spatial resolution of FCM is more macroscopic, related to the site of sampling. Only recently, hybrids between both technologies have become

available in the form of laser scanning cytometers and, perhaps in the long run, the restriction of image analysis to data processing of critical data only may lead us back to the microscopical beginning.

1.2 Flow cytometry and single cell sorting

In a flow cytometer cells, or other particulate matter, flow through a zone of investigation where parameters of interest are measured. The history of bacterial flow cytometry probably starts with the work of Tyndall in the mid 19th century. He detected the absence of particles in the air of his dust free box by means of light scattering in a light beam as illustrated in microbiology text books (e.g. Pelczar, Jr. *et al*. 1993). And nearly 200 years after the onset of cytometry by Leeuwenhoek, it was Robert Koch's manual cell sorting which led to the isolation of *Bacillus anthracis*, proving the link between a disease and a certain bacterium.

In modern flow cytometers the measurement is taken electronically. The classic example of FCM is the Coulter Counter, where cells are suspended in a particle free solution and a fixed volume is passed through a narrow orifice. Depending on their size, the particles change the electric current running across the orifice, generating signals which give rise to accurate enumeration and particle sizing. In the context of this study flow cytometry is restricted to instruments based on optical measurements. The major elements of such a modern multi parameter flow cytometer are shown in Figure 2. Typically, light scatter and fluorescence signals are measured to provide a variety of information on, for example, surface-structure, membrane permeability, pH, or DNA/RNA content. The fluidic system is designed to guide the cells in single file through the centre of a focused laser beam (hydrodynamic focusing). The amount of light scattered or emitted by each particle is measured, digitalized and fed into a computer. There the different optical signals are correlated and groups or clusters of cells are identified and statistically analysed as shown in Figure 3.

Certain instruments allow the user not only to analyse these cell populations but also sort them for preparative purposes. From all the sorting principles (Lindmo *et al*. 1990) the droplet-based sorters have become the most widespread systems. In those sorters the flow chamber vibrates vertically at a high frequency and the out-coming liquid stream is disrupted into small uniform droplets. At a fixed time after the cell is measured it reaches the last droplet attached to the liquid stream. If the cell falls in a cluster of interest, it is then selected for sorting and the liquid stream is charged positively or negatively for the time of droplet separation. Depending on the charge, the droplet is deflected in an electric field into collection vessels for subsequent analysis.

The strength of flow cytometry lies in its capacity for single cell measurements, its acquisition speed and its numerical power. The total illumination of the particle in the laser beam allows the quantification of the fluorescence intensity per particle. By looking at multiple parameters of a thousand cells per second, groups or clusters can be identified. Screening several thousand cells also allows the detection of low frequency events with a statistical significance. Correct total enumeration of aerobic, anaerobic and facultative anaerobic bacteria in mixed populations becomes possible, as the method does not depend on post sampling growth.

The most detailed descriptions of flow cytometric systems, including how to build your own, can be found in "Practical Flow Cytometry" by Howard Shapiro (Shapiro, 1995). It also represents the most comprehensive source about staining techniques which can be applied. Flow Cytometry and Sorting (Anonymous1990a) also gives detailed technical background on flow cytometry and there are other extensive manuals such as "Current Protocols", "Flow Cytometry" as part of "Methods in Cell Biology" (Anonymous1994) that cover various aspects of the technology. The handbooks of Longobardi-Givian (Longobardi-Givian, 1992), Ormerod (Anonymous1990b) and particularly the manual published by the Royal Microscopical Society (Ormerod, 1994) might serve as a more easy to read literature for beginners that focus on the essential concepts.

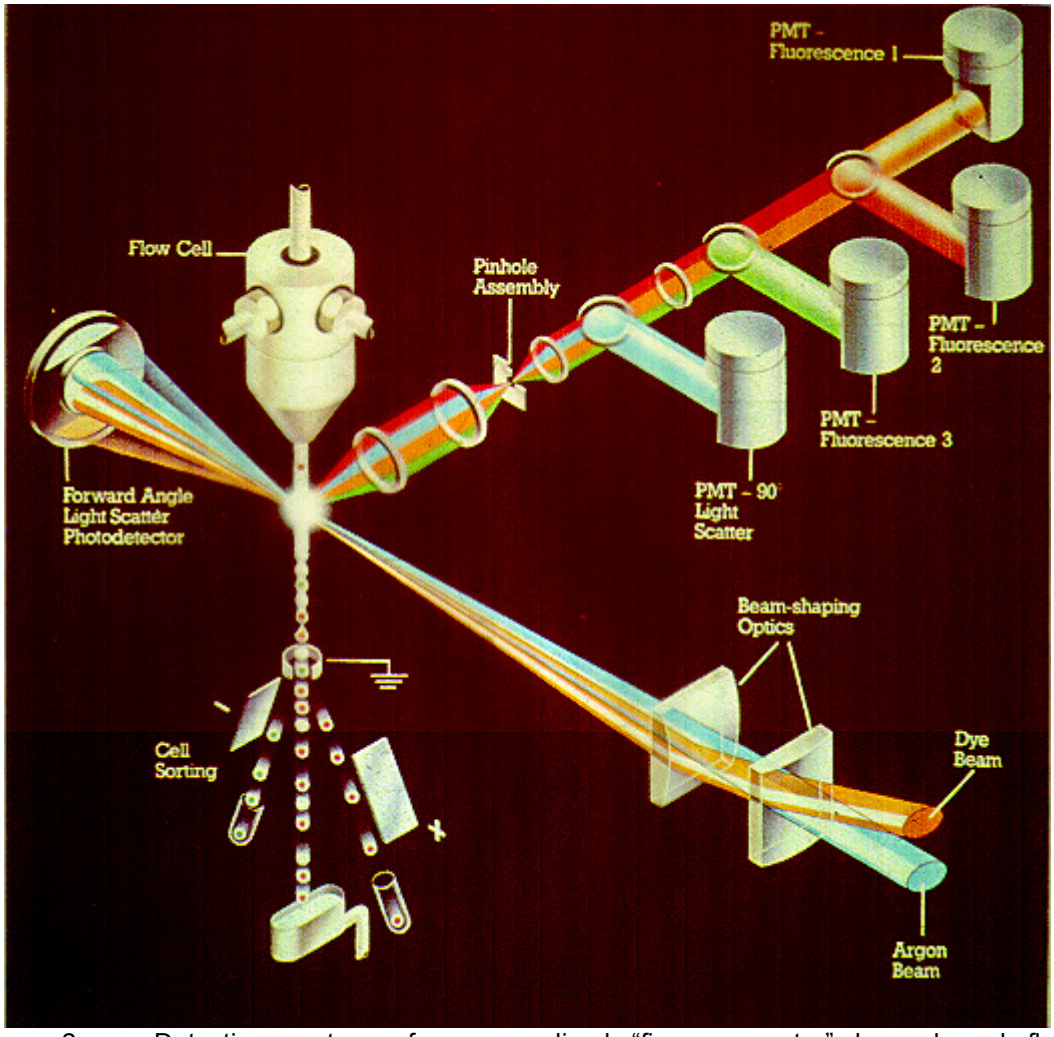
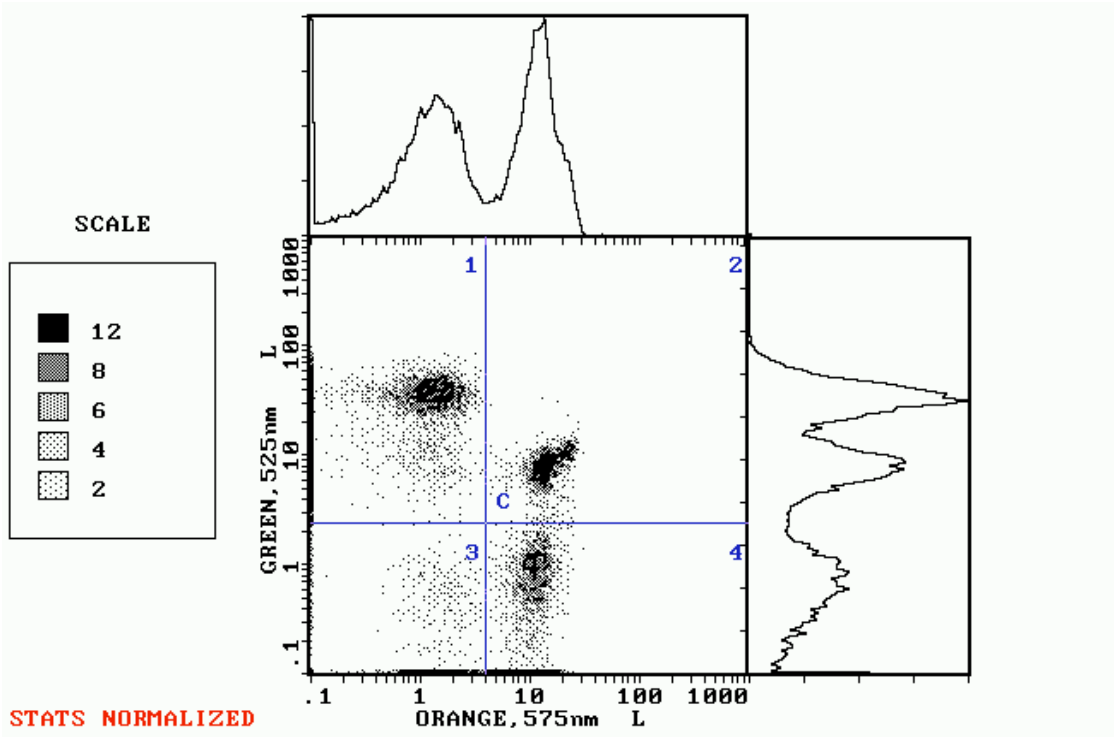


Figure 2: Detection system of a generalised "five parameter" laser based flow cytometer

A sheath flow is running through a flow cell forming a laminar liquid stream. Into this stream a particle or cell suspension is injected to be guided into a sensing zone in single file, one after the other. Whenever a cell or particle goes through the intercept with the illuminating laser beam, light is scattered. Photons of the same wavelength as the incoming light are collected axial and perpendicular to the light beam (forward angle and right angle light scatter). Fluorescent signals are also collected perpendicular to the light beam and separated onto different detectors using mirrors and filters with appropriate spectral characteristics. The photomultiplier tubes (PMT's) convert the light intensity into electric signals that are fed into a computer. Cell sorting is achieved by vertically vibrating the flow cell at several thousand hertz to generate uniform droplets. If an event fulfils the desired scatter and fluorescent properties, the whole liquid system is charged with a high voltage when this cell has reached the point of droplet breakoff. Depending on the given charge, the droplet containing that cell can therefore be deflected in an electric field and deposited in tubes, on slides or agar plates.



ID	%	Count	Mn X	MnI X	Md X	SD X	Mn Y	MnI Y	Md Y
C1	44.0	8806	0.569	1.01	0.810	0.70	21.4	29.1	27.5
C2	16.2	3231	13.7	15.4	14.0	5.85	7.24	9.12	7.39
C3	14.7	2947	0.936	1.43	1.28	1.03	0.349	0.552	0.372
C4	25.1	5016	10.3	11.5	10.5	4.10	0.455	0.702	0.553

Figure 3: Data analysis of a two parameter or bivariate dot plot

The figure shows a typical analysis screen of the Coulter Version II software. The display is a correlation of orange versus green fluorescence on the projection of the single channel histograms. Increasing dot density represents increasing number of particles with similar measurement values, thus clustering. Whilst the single parameter histograms projected to the sides already indicate two or three populations contained in the sample, the true heterogeneity only becomes apparent when correlating separate parameters. The clusters are then analysed by regions of interest for relative and absolute counts and signal intensity as shown at the bottom of the screen.

1.3 Historical background

The history of cytometry of single microbes goes back to the discovery of the 'animalcules' by Leeuwenhoek with his microscope who made drawings to characterise their morphology, followed by Koch who already used photography to document his microscopic observations down to modern image analysis systems. Flow cytometry probably started with the 'dust free box' of Tyndall in the late 19th century. He observed the light scattering of aerosols in the path of a light beam in order to determine the stage at which he could expose broth to the air without becoming contaminated. Driven by the need to identify bacterial aerosols in warfare, the next generation of flow cytometers started a mere 100 ears later, with a similar design in the late 1940's (Gucker et al. 1947; Ferry et al. 1949; Gucker and O'Konski, 1949). The next period of more intensive flow cytometry in microbiology started in the mid 1970's by Hutter (Hutter, 1974; Hutter et al. 1975a; Hutter et al. 1975b); Paau *et al* (1977); Slater *et al* (1977) and Bailey *et al* (1977). Hutter and Eipel (1978) were the first to undertake a complex study on viability, total protein and cell cycle of bacteria, yeast and moulds and the auto-fluorescence of algae. They already utilised the power of multiparameter measurements possible with FCM, a feature neglected in most of the more recent studies. In 1980 Hutter also started to apply the technique to look at bacterial growth inhibition (Hutter and Oldiges, 1980). At the same time Steen used a modified microscope which he developed into a flow cytometer more geared for microbial

applications (Steen and Lindmo, 1979; Steen and Boye, 1980; Steen, 1983). He did fundamental work in bacterial replication and subsequently drug susceptibility (Steen et al. 1982; Steen et al. 1986) and also applied immunofluorescence (Steen et al. 1982). Further work in flow cytometric differentiation by antibody staining was done by Ingram *et al* (Ingram et al. 1982), Sahar *et al* (Sahar et al. 1983), Phillips and Martin (Phillips and Martin, 1983; Phillips and Martin, 1985), Barnett *et al* (Barnett et al. 1984) and Libertin *et al* (Libertin et al. 1984). Since the eighties, the number of articles applying FCM in microbiology seems to follow exponential growth.

Successful cell sorting of bacteria was probably first described by Paau *et al* (Paau et al. 1979) who separated algae from bacteria. Other early papers were Cohen *et al* (Cohen et al. 1982), Libertin *et al* (Libertin et al. 1984) and the technique has been exploited extensively in the industry for strain improvement (Betz et al. 1984). Libertin *et al* were the first to use sorting in combination with immunofluorescence for the detection of *Pneumocystis carinii* for microscopical confirmation of the organism, a principle revisited nearly ten years later for the analysis of *Cryptosporidium* (Vesey et al. 1993).

Flow cytometry has become one of the key techniques in analytical cytology of mammalian cells. The success of the technique for example in the field of clinical immunology is based on three main factors:

0. the big separation between differentiated clusters that allows easy data interpretation
1. the functional significance of these clusters
2. the positive attitude of medical scientists towards technology

The application of the technique in microbiology clearly represents a challenge as compared to mammalian cells, bacteria are only 1/10 of the diameter, thus cell surface is only 1/100 and cell volume 1/1000 which has clearly implications on the signals derived from them. The acceptance of the technology is growing as well in the field of microbiology. This is partially due to the improvements in the technology, leading to data and cluster separation that start to become convincing even to non flow cytometrists. To gain even more acceptance, it is necessary to resolve some of the conflicting data with regards to the applicability of fluorescent labelling and to verify functional significance of clusters as determined by flow cytometry by means of sorting.

References:

Bailey, J.E., Fazel-Madjlessi, J., McQuitty, D.N., Lee, Y.N., Allred, J.C. and Oro, J.A. (1977) Characterization of bacterial growth by means of flow microfluorometry. Science 198, 1175-1176.

Barnett, J.M., Cuchens, M.A. and Buchanan, W. (1984) Automated immunofluorescent speciation of oral bacteria using flow cytometry. J. Dent. Res. 63, 1040-1042.

Betz, J.W., Aretz, W. and Härtel, W. (1984) Use of flow cytometry in industrial microbiology for strain improvement programs. Cytometry 5, 145-150.

Cohen, J., Perfect, J.R. and Durack, D.T. (1982) Method for the purification of Filobasidiella neoformans basidiospores by flow cytometry. Sabouraudia. 20, 245-249.

Ferry, R.M., Farr, R.M., Jr. and Hartman, M.G. (1949) The preparation and measurement of the concentratoins of dilute bacterial aerosols. Chem. Rev. 44, 389-417.

Gucker, F.T., O'Konski, C.T., Pickard, H.B. and Pitts, J.N. (1947) A photoelectronic counter for coloidal particles. J. Am. Chem. Soc. 69, 2422-2431.

Gucker, F.T. and O'Konski, C.T. (1949) Electronic methods for counting aerosol particles. Chem. Rev. 44, 373-388.

Hutter, K.J. (1974) Untersuchungen über die DNS-, RNS- und Proteinsynthese von Hefezellen der Gattung Sacharomyces mit Hilfe neuer fluorometrischer Methoden. 19/FB13, Berlin.

Hutter, K.J., Boose, H., Oldiges, H. and Emeis, C. (1975a) Investigations about the synthesis of DNA, RNA and proteins of selected populations of microorganisms by Cytophotometry and Pulse-Cytophotometry. 3. Determination of intracellular substancesof partially synchronized aerobe and respiratory deficient yeast cells. (Untersuchungen über die DNS-, RNS- und Proteinsynthese ausgewählter Mikroorganismenpopulationen mit Hilfe der Zytophotometrie und der Impulscytophotometrie. 3. Mitteilung: Bestimmung der Zellinhaltsstoffe teilsynchronierter atmungsfähiger und atmungsdefecter Hefezellen.). Chem,Mikrobiol. Technol. Lebensm 4, 101-104.

Hutter, K.J., Göhde, W. and Emeis, C. (1975b) Investigations about the synthesis of DNA, RNA and proteins of selected populations of microorganisms by Cytophotometry and Pulse-Cytophotometry. 1.Methodical investigations about appropriate fluorescence dyes and staining procedures. (Untersuchungen über die DNS-, RNS- und Proteinsynthese ausgewählter Mikroorganismenpopulationen mit Hilfe der Zytophotometrie und der Impulscytophotometrie. 1. Mitteilung: Methodische Untersuchungen über geeignete Färbeverfahren.). Chem,Mikrobiol. Technol. Lebensm 4, 29-32.

Hutter, K.J. and Eipel, H.E. (1978) Flow cytometric determinations of cellular substances in algae, bacteria, moulds and yeasts. Antonie van Leeuwenhoek 44, 269-282.

Hutter, K.J. and Oldiges, H. (1980) Alterations of proliferating microorganisms by flow cytometric measurements after heavy metal intoxication. Ecotoxicol. Environ. Saf. 4, 57-76.

Ingram, M., Cleary, T.J., Price, B.J., Price, R.L. and Castro, A. (1982) Rapid detection of Legionella pneumophila by flow cytometry. Cytometry 3, 134-137.

Libertin, C.R., Woloschak, G.E., Wilson, W.R. and Smith, T.F. (1984) Analysis of Pneumocystis carinii cysts with a fluorescence- activated cell sorter. J. Clin. Microbiol. 20, 877-880.

Paau, A.S., Cowles, J.R. and Oro, J. (1977) Flow-microfluorometric analysis of Escherichia coli, Rhizobium meliloti, and Rhizobium japonicum at different stages of the growth cycle. Canadian Journal Of Microbiology 23, 1165-1169.

Paau, A.S., Cowles, J.R., Oro, J., Bartel, A. and Hungerford, E. (1979) Separation of algal mixtures and bacterial mixtures with flow-microfluorometer using chlorophyll and ethidium bromide fluorescence. Archives Of Microbiology 120, 271-273.

Phillips, A.P. and Martin, K.L. (1983) Immunofluorescence analysis of bacillus spores and vegetative cells by flow cytometry. Cytometry 4, 123-131.

Phillips, A.P. and Martin, K.L. (1985) Dual-parameter scatter-flow immunofluorescence analysis of Bacillus spores. Cytometry 6, 124-129.

Sahar, E., Lamed, R. and Ofek, I. (1983) Rapid identification of Streptococcus pyogenes by flow cytometry. Eur. J. Clin. Microbiol. 2, 192-195.

Slater, M.L., Sharrow, S.O. and Gart, J.J. (1977) Cell cycle of Saccharomycescerevisiae in populations growing at different rates. Proc. Natl. Acad. Sci. U. S. A. 74, 3850-3854.

Steen, H.B., Boye, E., SKARSTAD, K., Bloom, B., Godal, T. and Mustafa, S. (1982) Applications of flow cytometry on bacteria: cell cycle kinetics, drug effects, and quantitation of antibody binding. Cytometry 2, 249-257.

Steen, H.B. (1983) A microscope-based flow cytophotometer. Histochem. J. 15, 147-160.

Steen, H.B., SKARSTAD, K. and Boye, E. (1986) Flow cytometry of bacteria: cell cycle kinetics and effects of antibiotics. Ann. N. Y. Acad. Sci. 468, 329-338.

Steen, H.B. and Boye, E. (1980) Bacterial growth studied by flow cytometry. Cytometry 1, 32-36.

Steen, H.B. and Lindmo, T. (1979) Flow cytometry: a high-resolution instrument for everyone. Science 204, 403-404.

Vesey, G., Slade, J.S., Byrne, M., Shepherd, K., Dennis, P.J. and Fricker, C.R. (1993) Routine monitoring of Cryptosporidium oocysts in water using flow cytometry. J. Appl. Bacteriol. 75, 87-90.

2 Technical background

2.1 Setting the environment :

2.1.1 Requirement on labware and reagents.

Measuring bacteria means detecting submicron particles. Therefore it is essential to ensure that all reagents are not only sterile but also particle free. That means that all solutions have to be passed through at least 0.45 μm or better 0.2 μm filters.

Labware should also be dust and particle free. The major problem of washed glassware is the accumulation of paper fibres from autoclave tape, usually not removed prior to washing. Therefore sterile filtration should be performed into disposable labware if possible. Safety considerations in particular with regards to the handling of infectious and potentially carcinogenic material also suggest the use of plastics.

The reagents used should be analytical research grade (AnalaR) where possible. Buffer solutions and liquid media should regularly be checked for pH and osmolarity as a form of basic quality control in particular after addition of ingredients (like for example EDTA).

2.1.2 Preparation and handling of dye solutions

With respect to health and safety regulations, at least the DNA fluorochromes have to be treated as mutagenic. With the risk posed by the other dyes with unknown toxicological properties and the solvents used, it should be common practice to treat all dye solutions as potentially carcinogenic. Thus it is important to wear gloves, labcoat and if required protective eye-wear. Dry components should be handled in a draft free environment to avoid the formation of dust. Work areas should be wiped generously with alcoholic solutions like 75% isopropanol prior and after handling the dyes. Sterile filtration of dye solutions should be performed by centrifugation through filter membranes like for example 0.2 mm Anapore Micro-Centrifuge tube filters (Whatman, Maidstone, UK) to avoid the risk of spluttering.

Disposal of stained samples has to be done by incineration to achieve destruction of the potential carcinogens. Autoclaving does not destroy the compounds and sample liquids would pose a risk to service personnel. The waste liquid from the cytometer should be treated with sodium hypochlorite (>2500 ppm) over night and neutralised with Sodium thiosulfate before disposal.

If possible, the use of solvents should be avoided. They can change the membrane permeability to the dyes and other molecules and can either distort the measurements or even be cytotoxic. They can also sometimes penetrate laboratory gloves and increase the risk of dye handling.

Please note that the concentrations below are guiding figures and should always be optimised for the test conditions. There can be numerous components in growth media can severely reduce the amount of freely available fluorochromes. Apart from Molecular Probes, dyes can be sourced from Lambda Fluorescence Technology, Polysciences and Eastman-Kodak.

Table 1
Commonly used dye solutions: commercial sources, solvents and concentrations.

BOX	Bis-Oxonol or bis-(1,3-dibutylbarbituric acid)trimethine oxonol ($DiBAC_4(3)$)		
	[Molecular Probes, Eugene, OR, USA, # B-438] (FW 516.64)		
	Stock solution :	10.0	mg/ml in DMSO, -20°C
	Working solution :	10 or 100.0	μg/ml in A.D., 0.5% Tween, 4°C
	Final concentration :	0.1-1.0	μg/ml
	(Oxonols may require addition of a base to be soluble.)		
EB	Ethidium Bromide		
	[Sigma, Poole, UK; # E8751] (FW 394.3)		
	Stock solution :	10.0	mg/ml in A.D., -20°C
	Working solution :	500.0	μg/ml in A.D., 4°C
	Final concentration :	5-10.0	μg/ml

PI	Propidium Iodide		
	[Sigma, Poole, UK, # P4170] (FW 668.4)		
	Stock solution :	2.0	mg/ml in A.D., 4°C
	Working solution :	500.0	μg/ml in A.D., 4°C
	Final concentration :	5-10.0	μg/ml
RH123	Rhodamine 123		
	[Lambda Fluoresce Technologie, Graz, Austria, # LP-250] (FW 380.8)		
	Stock solution :	10.0	mM in DMSO, -20°C
	Working solution :	0.1	mM in A.D., 4°C
	Final concentration :	0.2-1.0	μM
CFA	Carboxy-Fluorescein-diAcetate		
	[Lambda Fluoresce Technologie, Graz, Austria, # LA-551] (FW 460.4)		
	Stock solution :	10.0	mM in DMSO, -20°C
	Working solution :	100.0	μM in A.D., 4°C
	Final concentration :	20-50.0	μM
CCFAS	diChloro-CFA-Succinimidylester		
	[Lambda Fluoresce Technologie, Graz, Austria, # LA-574] (FW 626.37)		
	Stock solution :	10.0	mM in DMSO, -20°C
	Working solution :	100.0	μM in A.D., 4°C
	Final concentration :	20-50.0	μM
Bac Light	proprietary mixture of fluorochromes		
Viability Kit	[Molecular Probes, Eugene, OR, USA, #		

2.1.3 Sample Handling

Apart from the chemical hazard already mentioned, bacterial samples bare a biological risk. The two key steps in the sample handling process were bacteria can become airborne are the mechanical sample disaggregation and the measurement in the cell sorter. Thus in the case of sample sonication it is important to use lids on tubes or vessels and to ensure that sonicator probes are sufficiently submerged into the liquid. Single cell sorting requires the formation of small droplets to be deflected in an electric field. The sort chamber of the EPICS Elite already forms a biohazard containment incorporating a screen protection for the operator and the application of a slight under-pressure to the sort chamber, sucking air through a biohazard filter. 'Mist' formation occurs either when the sort crystal is out of tune or the sort stream hits a horizontal surface. Both can be prevented by careful alignment of the system prior to the measurement of samples and is required for successful sorting anyhow.

Whilst all lab solutions require filtration to reduce background signals, samples also require filtration to protect flow instrumentation from becoming clogged. As any 77 μm particle is bound to block the 76 μm sort nozzle it is recommended to pre-filter in particular environmental samples through a 50 μm nylon filter mesh. Whilst it is possible to obtain such material in bulk sheets (Züricher Beuteltuchfabrik, Rüschlikorn, Switzerland) ready to use devices are nowadays available as sterile disposable units from a number of companies (Dako, High Wycombe, UK; Partec, Münster, Germany). In addition the sample inlet of sorters should also be fitted with a piece of filter mesh. As this can increase the risk of sample carryover it is advisable to run a sterile filtered detergent containing 'wash-sample' between samples.

2.2 Setting up the instrument : Calibration standards, Discriminator settings

The initial and daily instrument alignment should be made with a three or more bead mixture of small fluorescent (yellow/green) and nonfluorescent latex beads around >300nm, 600nm and 1000nm. The smaller the particles the lesser they follow the hydrodynamic focusing. Usually optimal alignment requires volume flow rate settings close to flow cut off.

Select a display of log side scatter (y-axis) versus log green fluorescence (x-axis). As most instruments have a very high sensitivity on the green fluorescence signal, discriminator (or threshold or trigger) are usually first set onto green fluorescence running a blank sample. The discriminator is lowered to a background count of 50 to 100 events per second. Running the bead sample should the generate clusters that will allow to set the instrument voltages to resolve the clusters. As a guideline the ≈600nm YG fluorescence of Polysciences beads should be in the 4th decade. Whilst the fluorescent distribution depends on your chosen beads, the light scatter distribution should

have a spread similar to Figure 4, the 500nm beads approximately mid scale. Once that dot plot is achieved, detection can be switched to scatter half scale (500) and decreased slowly. In general discrimination on peak signals results in less noise. When scatter gating is activated noise (and nonfluorescent beads of similar size should spread across the first decade of the green fluorescence.

An improvised method to check out an instrument is using some late exponential bacteria and heatfix them by just boiling the broth. Adding 10 μl of that sample to 2 ml PBS containing 1 $\mu g \times ml^{-1}$ PI usually allows to set up the instrument based on red fluorescence versus scatter as described above, but that method does not replace a proper calibration.

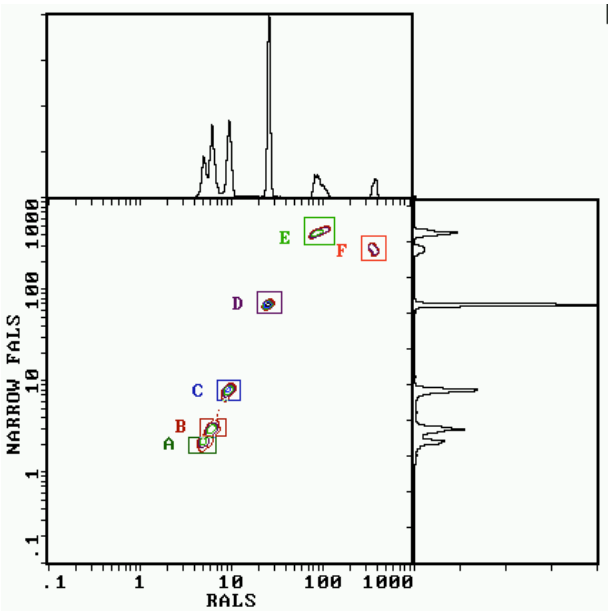


Figure 4
The distribution of latex beads at different light scatter angles: The alphabetical order of the regions from A to F in the contour plots matches the increasing diameter of 350 nm, 380 nm, 500 nm 660 nm, 1160 nm and 2230 nm. The relative positions already indicate non-linearity between narrow FALS and bead diameter.

2.3 *Signal processing: Bacterial discrimination, back-gating*

Using clean beads, discrimination on fluorescence gives a pretty clean picture. That is more likely to occur when triggering on scatter. The main sources of interference are particulate matter in sheath or sample in form of precipitation which require filtration. In case of the sheath fluid it is important to have a sterile filter close to the flow cell, as particles are released from aged tubing. Noise can be generated from dust stuck in the light path increasing the constant photon background leading to a noisier detector, and little pieces of dust can even generate modulated photon flow.

To familiarise yourself with instrument performance it is a good idea to play around with 'fat cells' from a culture. At high enough concentrations interference problems with non bacterial events are rare. At lower concentrations the biggest contribution of non-bacterial matter is usually the sample. This can be precipitates from the growth medium or micelles or precipitates that form as a result of substances added to the sample like antibiotics. Thus it is important to be able to discriminate bacteria from non-bacterial matter. Apart from using supravital DNA stains this is best achieved by analysing cluster formation in parameters independent from the fluorescent plot under investigation (Figure 5). As modern cytometry software packages are more powerful, it is a good idea to set up one set of displays of scatter (y-axis) versus fluorescence (x-axis) to look at data clustering (see Figure 5c).

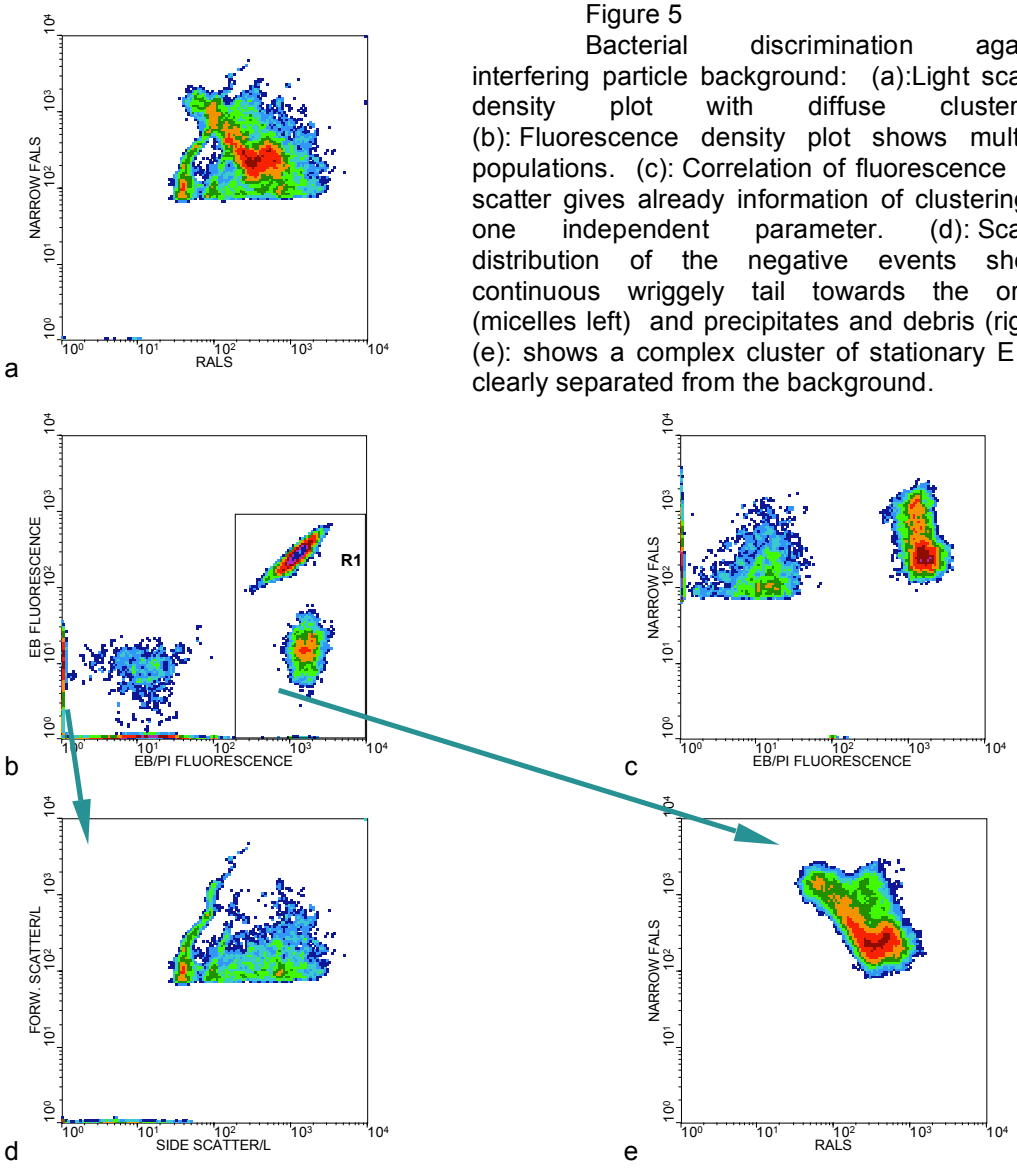


Figure 5
Bacterial discrimination against interfering particle background: (a):Light scatter density plot with diffuse clustering. (b): Fluorescence density plot shows multiple populations. (c): Correlation of fluorescence and scatter gives already information of clustering in one independent parameter. (d): Scatter distribution of the negative events shows continuous wriggly tail towards the origin (micelles left) and precipitates and debris (right). (e): shows a complex cluster of stationary E.coli clearly separated from the background.

2.4 Light Scatter measurements : Opportunities and limitations

Detection sensitivity by light scatter is limited by the optical arrangement. Reports about the correlation between scatter measurement and cell volume are conflicting (Allman et al. 1993; Boye and Steen, 1993). Whilst the correlation between RALS and particle cross section reached r^2=0.9997, Figure 6 already demonstrates that the situation is more complex. The bacteria appear in the same narrow FALS area as the 500nm beads (Figure 6a, Y-axis), but lower than the 350nm particles in RALS (Figure 6b, X-axis). In the end this is not too surprising as, apart from particle size, scatter signals depends on cell shape, refractive index and extinction coefficient. But still, the information obtained represents an important information to indicate changes in morphology and correlation can be obtained on pure cultures in short term observations were changes in refractive index, OD and aggregation are not critical (Davey et al. 1993; Durodie et al. 1993; Wold et al. 1994).

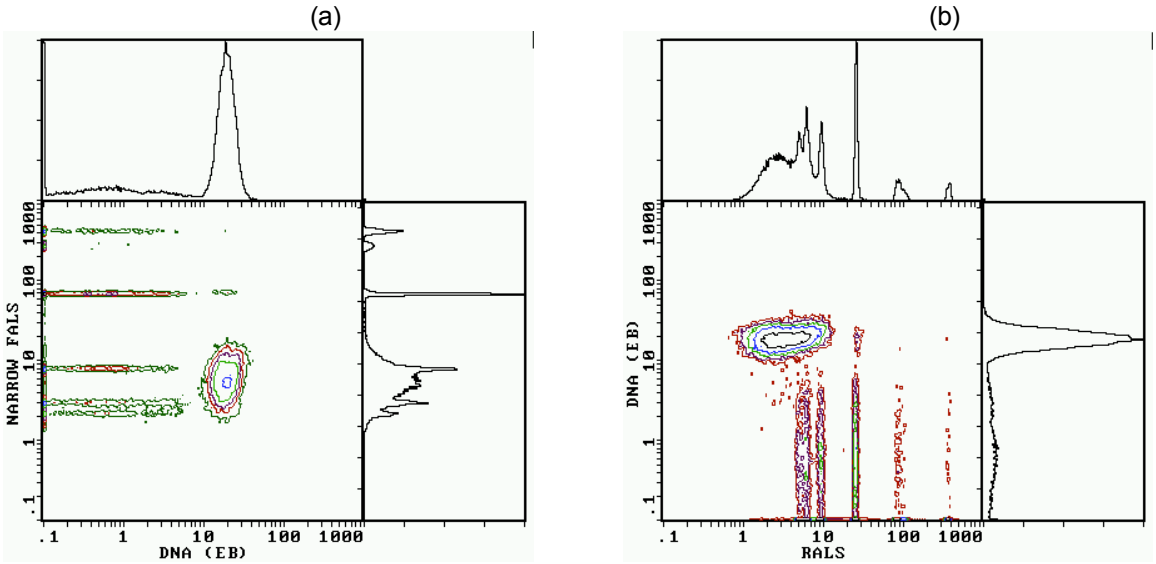


Figure 6

Light scatter signal of *Peptostreptococcus anaerobis* stained with ethidium bromide in comparison to a range of latex beads

The limitation in scatter sensitivity becomes more critical when looking at particles as small as elementary bodies of *Chlamydia trachomatis*. This is demonstrated in Figure 7, where discrimination on scatter only did not resolve the particles of interest. This is another example demonstrating the importance of multichannel discrimination.

As already mentioned in the previous section, backgating on scatter can help confirming populations. It also can help to achieve cluster differentiation as for example with the spores in Figure 8 or as described by Allmann *et al*. potentially together with stains for DNA or membrane potential for species differentiation under controlled growth conditions (Allman et al. 1993).

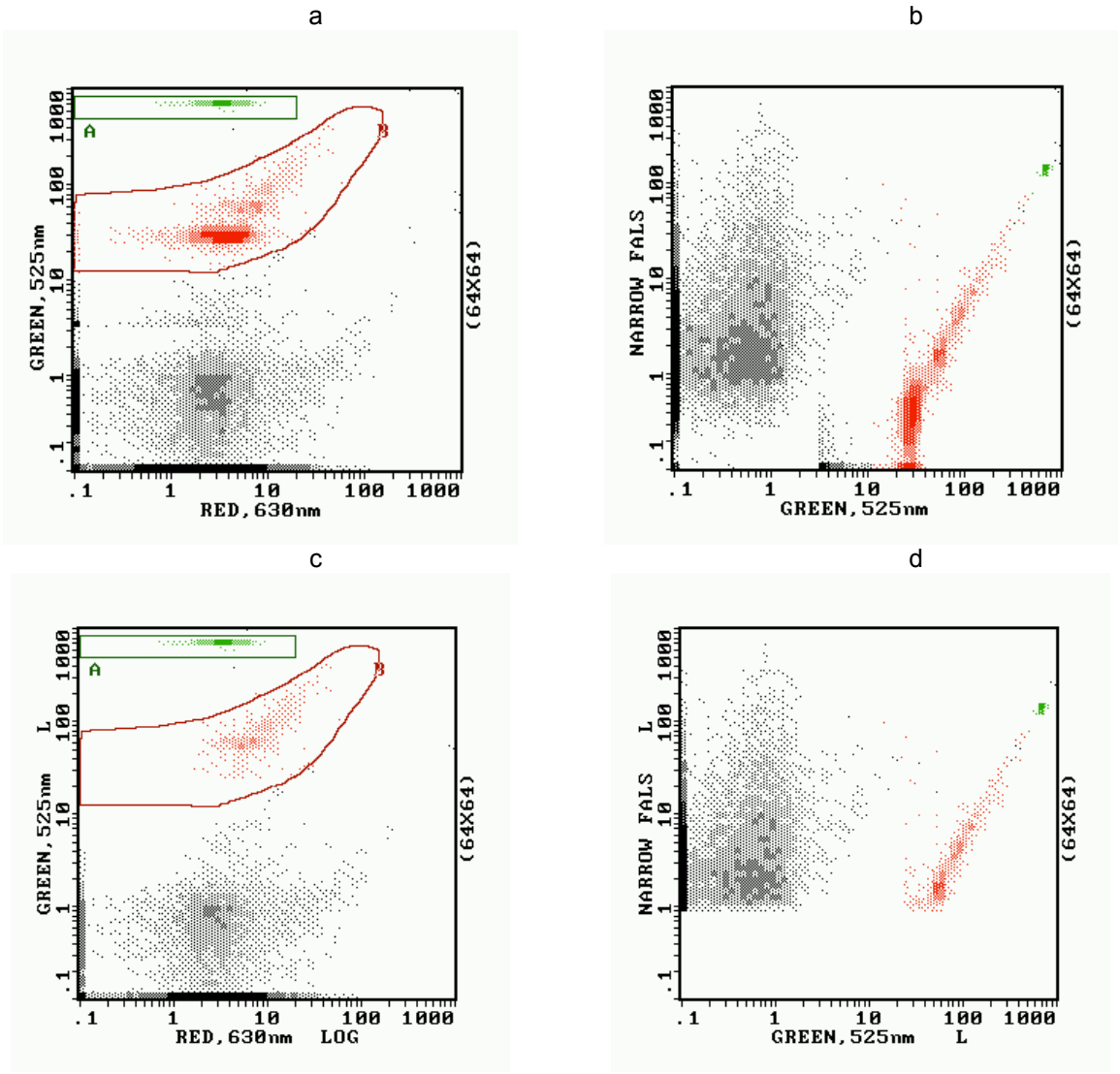
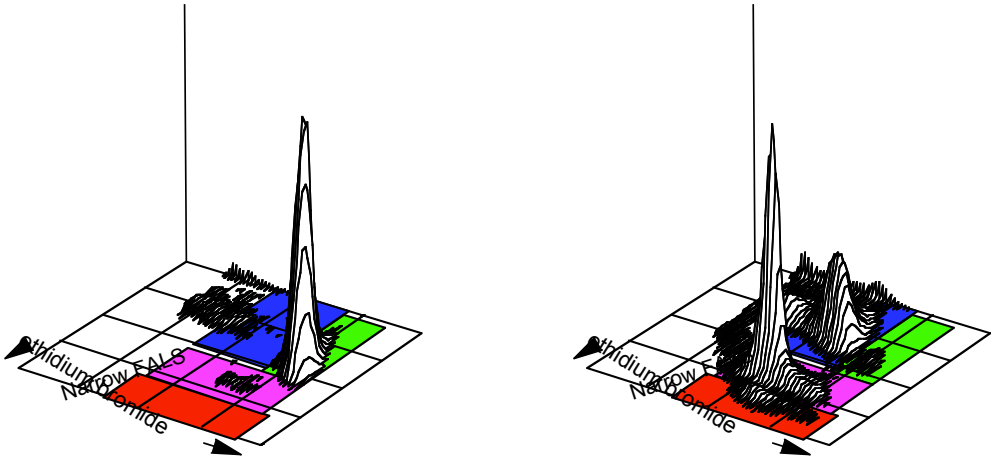


Figure 7

The requirement for multiparameter thresholding for the detection of *Chlamydia trachomatis* : Culture supernatants of C.trachomatis infected McCoy cells were incubated with FITC labeled antibody as described in 3.4.4. Detection was based on the simultaneous discrimination on both, FALS and green fluorescence. Threshold or trigger-levels were set to produce a background detection rate of less than 50 counts per second. Elementary bodies in picture a of green antibody fluorescence (Y-axis) versus red DNA stain (X-axis) are highlighted by region B. The DNA signal of the smallest population was equal to the background noise (bottom left cluster). In picture b this population lies also below the noise threshold of the forward scatter (Y-axis) of channel 1. The effect of discrimination of forward scatter only is shown for the same sample in pictures (c) and (d). The discrete populations of the elementary bodies creates the false impression that all the elementary bodies are detected, as the smallest detected cluster is separate from the background.

a: 5 minutes germination

b: 60 minute germination



c: 90 min germination

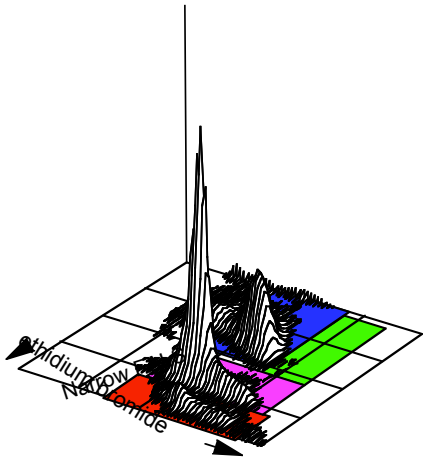


Figure 8
Germination of *Bacillus cereus* observed by ethidium bromide staining: Spores from a frozen preparation in distilled water were diluted in Brain Heart infusion and kept at room temperature. Samples were taken into DBS_{AT} immediately, after 60 and 90 minutes, sonicated for 30 seconds in the hot spot of an ultrasonic waterbath and stained with $5\mu g \cdot ml^{-1}$ EB. The isometric display has the origin in the top corner. The Y-axis (pointing to the right) represents Narrow FALS and the X-axis (pointing to the left) the red fluorescence of the EB. Initially most spores form a uniform cluster in the green region with only very few EB positive events (pink region). The blue region represents debris (a) and capsular material separated from the spores by the sonication (b,c). Whilst the germinating spores initially decline in FALS signal, the signal increases again together with an increase in EB fluorescence.

2.5 Sorting bacteria : Instrument preparations and sorting

In principle the same as for mammalian cells, only that you can use antibiotics as a back up to prevent contamination. As some commercial sheath liquids contain antimicrobials, standard buffers should be used as sheath liquid. The other main consideration is the handling / sorting of biohazard material which has been recently adressed by ISAC (Schmid, 1997)

The installation of self-bleeding disposable filter cartridges (Millex GV, Millipore, Bedford Massachusetts, USA) in close proximity of the flow cell is the most valuable adaptation. As filters can break through and become contaminated backwards through the pipework, the cartridges are replaced on any sorting day. When vacuum is applied to the flow cell, the remaining sheath line can be cleaned very effectively by sucking up 50ml of a 50% Domestos solution (1:1 with distilled. water), as there are no pressure sensors or dead spaces between the filter connections and the flow cell. The luer connectors are then rinsed with 10ml 75% isopropanol also sucked through the pipe and a new filter is attached and labelled with a date. The sample line is also treated by running 2ml of 50% Domestos followed by an isopropanol rinse. To rinse the sample rod between samples with distilled water, a 50ml syringe with an end point sterile filter is used.

!! For reasons of fire-safety it is important to ensure that the high voltage deflector plates are switched off all the time whilst instrument disinfection takes place.!!

The handling surfaces in front of the instrument and the sorting area are also sprayed generously with 75% isopropanol, including the deflection plates, and excess liquid is wiped away.

To minimise the risk of splattering droplets, which can give rise to aerosol formation, it is important to ensure that the waste stream of the system hits the waste collector at the sloped edge. When hitting a surface perpendicular to the droplet flow, splattering is more likely to occur. Foam in the waste collector can also lead to splattering. It can be removed by a drop of ethanol delivered with a pasteur pipette.

After all that preparation contamination is less likely to come from the instrument but from handling the broth or agar plates. Therefore it is useful to have a cover above sort compartment and to leave lids on plates and Petri dishes until the sort starts. As the Autoclone® in the Elite moves the plates further then the sort compartment, a lid or cover should also be installed above that moving space. Use of gloves and intermittent disinfection of the hands can also reduce the risk if contamination.

Whilst the Autoclone could easily be adapted to take Petri dishes, their use for growth support can only be recommended for non-injured cells. Sorting 3nl liquid is not enough to wet the plate sufficiently, thus giving rise to additional osmotic and oxidative stress. However it gives an excellent opportunity to check for contamination by location of the sorted colony or more accurate enumeration if more than one cell is sorted as up to three cells / colonies can usually be distinguished. In addition one can obtain basic differentiation and use them to convince the microbiologists that what was a blip on the screen gives rise to their beloved little colonies. 96 well plates allow much better recoveries but detection of contamination is merely impossible.

Independent from the use of dish or plate, it is important when sorting subpopulations to have a selection of each subpopulation on every plate. This avoids falling for sorting drifts or changes occurring in the culture.

3 Functional and differential labelling of bacteria

3.1 Bacterial enumeration: Sample handling, disaggregation and counting methods.

All microbial detection systems that rely on cell replication are limited by our ability to get bacteria to grow. In natural populations symbiotic partners can be required or anaerobe micro-environments might exist that prevent accurate plate counts as some cells will only grow anaerobe, some only aerobe and some under both conditions. Thus correct counts, which detect healthy, injured, dormant, 'viable but sometimes-non-culturable' as well as truly dead bacteria can only be obtained by direct optical methods.

The major obstacle is to differentiate the bacteria from other debris in a sample. This is best achieved by DNA stains in combination with light scatter measurements. Special care has to be taken to avoid interference by DNA fragments and micelles that can pick up the dyes non-specifically. Double labelling with two fluorochromes of different membrane permeability allows more stringent bacterial discrimination of 'DNA surrounded by an intact cell membrane'.

Because of the counting error ($n^{0.5}$) it is necessary to investigate 100 events to achieve 10% variation. At 1000 events the coefficient of variation reaches 3% and above 5000 it goes below 1%. Thus because of the speed of analysis and the degree of automation, flow cytometry is preferable to image cytometry for enumeration unless spatial information is required.

3.1.1 Counting methods

- Fixed volume counting or volume integration :

This method is used in most haematology analysers. The volume measurement is achieved by two contact electrodes acting as level sensors in a known geometric set-up (Partec CAII) or by loading a cavity in a ceramic valve (Abbot Cell Dyn 3000, Coulter XL) or a loop made of tubing as done in high pressure liquid chromatography instruments. In all cases all events within the volume are measured.

Count or events can be lost by system leaks in particular when handling small volumes, flow restrictions or because of electronic dead times.

- Time integration :

This approach is based on the assumption of a constant volume flow over time. It is best achieved by (syringe) pumps delivering the sample. Such systems are implemented in the Ortho Cytron absolute and the former Skatron Argus flow cytometer now Bio-Rad Bryte HS.

Limits are as above, pump speed instabilities and sample carry-over. If differential pressure is used to transport the sample additional implications can arise from restrictions in sample or sheath flow, pressure fluctuations and changes in liquid viscosity due to additives or temperature.

- Spiking with reference particles (ratiometric counting) :

Mixing known volumes of solutions of reference particles and unknown sample allows calculation of absolute counts from the measured particle ratio. This method can be used with all cytometers. It corrects for system dead times and the variations in sample delivery rates as discussed above. The tight cluster of beads also serves as an on line alignment control, particularly important when measuring environmental samples that are more likely to block the flow path. Ratiometric counting also allows a certain freedom of sample manipulation in terms of washing and dilution steps. Becton Dickinson, Ortho Diagnostics and Coulter Corporation have released counted bead standards for use in clinical immunology and the System II software of the Coulter XL® has already implemented this method to give a direct output in absolute counts. Manual calculation can be obtained following the formula below:

$$\text{Sample conc.} = \frac{[\text{ref. volume}] \times [\text{sample predilution}] \times [\text{sample count}] \times [\text{ref. concentration ml}^{-1}]}{[\text{sample volume}] \times [\text{ref. predilution}] \times [\text{ref count}]}$$

The high number of counts achieved by flow cytometry make the technique superior to others with the regard of counting accuracy. By careful pipetting technique and using 0.05% Tween 20 to avoid cell sticking we could achieve counting variations within 1% of the expected numerical counting error.

The detection sensitivity of optical systems is limited by the statistical abundance of an event and the signal intensity separating the event from background noise. To identify a cell cluster it is desirable to have at least 100 cells in it. Thus if there is one organism per μl in the final sample volume, 100μl have to be measured. Relative frequency is another limit to the measurement. To detect a log 3 reduction equivalent to an event frequency of 0.1% 100.000 events need to be screened to see 100 wanted cells. Good signal to noise ratio is therefore important, as, at lower relative frequency, the labelled cells are end up within the standard deviation of the unlabelled events.

Signal to noise ratio is also limiting the speed of measurement, as with increased sample / volume throughput the variations broaden but in particular the background fluorescence increases due to free fluorochrome. With flow rates currently operating around $10\mu l \times min^{-1}$ this leaves a practical sensitivity of approximately 10^3 within 10 minutes. Lower concentrations require patience or pre-enrichment by physical or biological means.

3.1.2 Sample disaggregation

Single cell suspensions are essential for any form of accurate counting. Aggregates only give rise to one single colony or event. If for example one cell in a triplet is positive for a dead cell marker the whole aggregate is registered dead but will grow when sorted onto agar plates. This is a potential problem with samples like skin flakes, as hundreds of bacteria can be attached to a single flake.

Cells can be dissaggregated by either chemical or mechanical methods. Mechanical methods have a broader application spectrum but can lead to problems with filamentous organisms. Shearing by needles leads to problems with clogging and is very tedious. Shearing with homogenisers is difficult with small volumes and causes problems with foaming and sample carryover.

Ultrasonic treatment is the most convenient method, but it is important to apply reproducible energy levels. The geometry of the set-up and the material of the sample container has to be taken into consideration. When using a probe, energy loss can occur by coupling to ice cold water surrounding the sample container or by air bubbles trapped at the bottom of pointed vessels. Transmittable energy in an ultrasonic waterbath is sensitive to the level of water, its temperature and dissolved gas as well as its cleanliness. Soft container materials like polypropylene do absorb the energy in both systems.

Figure 9 shows an optimum recovery of cells when using a two minute sonication time at a 2μm amplitude. From the decrease of total cells and the relative increase of permeabilised cells we can see that sonication times above 10 minutes cause destruction of intact cells. The decrease of plate counts also suggests that cell damage occurs. 1μm sonication appears to give a wider 'window of constant recovery, but is more difficult to set up and the longer sonication time required makes it less feasible in practice.

Despite the disaggregation caused by the pipetting of the samples, sonication generated on average an increase of counts by a factor of 4 to 7 for cytometric counts and 6 to 8 for plate counts of smooth surface plaque. Immunofluorescent detection of *Strep. sanguis* resulted in a 12.2 fold increase compared to a 6.3-fold increase in total counts demonstrating species specific variation in aggregation.

Light scatter distribution usually tightens upon sonication and background separation improves. Rod shaped Actinomyces did show no increased sensitivity to sonication, but care needs to be taken when looking at filamentous organisms. Looking at older plaque samples has already shown that mechanical disaggregation is not always sufficient to disaggregate samples. Sonication in the presence of stains can lead to uptake of membrane impermeable dyes like PI and loss of antibody fluorescence as well as flagella.

Protocol for counting using a single colour stain:

The following protocol was used for the investigations into the sonication effects:

Sonicator: 3mm exponential probe at 23 kHz (MSE Soniprep 150).
Instrument settings: 1 and 2 μm amplitude for various time length.
Sample container: Disposable polystyrene 7ml flat bottom containers sonicated against air. The probe tip was 5mm below the liquid surface of a 2ml sample.
Samples: 24 hour bacterial plaque scraped with a wooden applicator and dissolved in 5ml of Dulbecco's phosphate buffered saline (DBS) split into 2 volumes at 2ml. Cultured organisms were grown in supplemented brain heart infusion containing 1% sucrose (S.sanguis, Actinomyces A8).
Staining: 20μl sample were diluted at various time points into 80μl DBS. 20μl of that solution were mixed with 20μl 0.66μm yellow-green fluorescent beads (Polysciences, Warrington, PA, USA) at

$1\times10^{8}ml^{-1}$ in DBS_{AT} containing 0.1%azide and 0.05% Tween 20 for counting, 10μl ethidium bromide (EB) or propidium iodide (PI) at 1mg/ml (Sigma, Poole, UK) and 150μl DBS_{AT}. After 15 minutes the samples were diluted in 1.8ml DBS and measured in the EPICS XL flow cytometer, with the first tube (EB) giving total number of bacteria and the second tube (PI) reflecting the permeabilized fraction.

Limitations:
Under some conditions the DBS can cause precipitation of sample components. If other buffers are used it is important to include the azide and Tween to inhibit dye extrusion and cell sticking. Azide is not always sufficient to block dye extrusion, thus needs to be tested for the particular application. Mild heat (45°C) (Sahar et al. 1983) or storage on ice (Jernaes and Steen, 1994)can facilitate EB uptake.

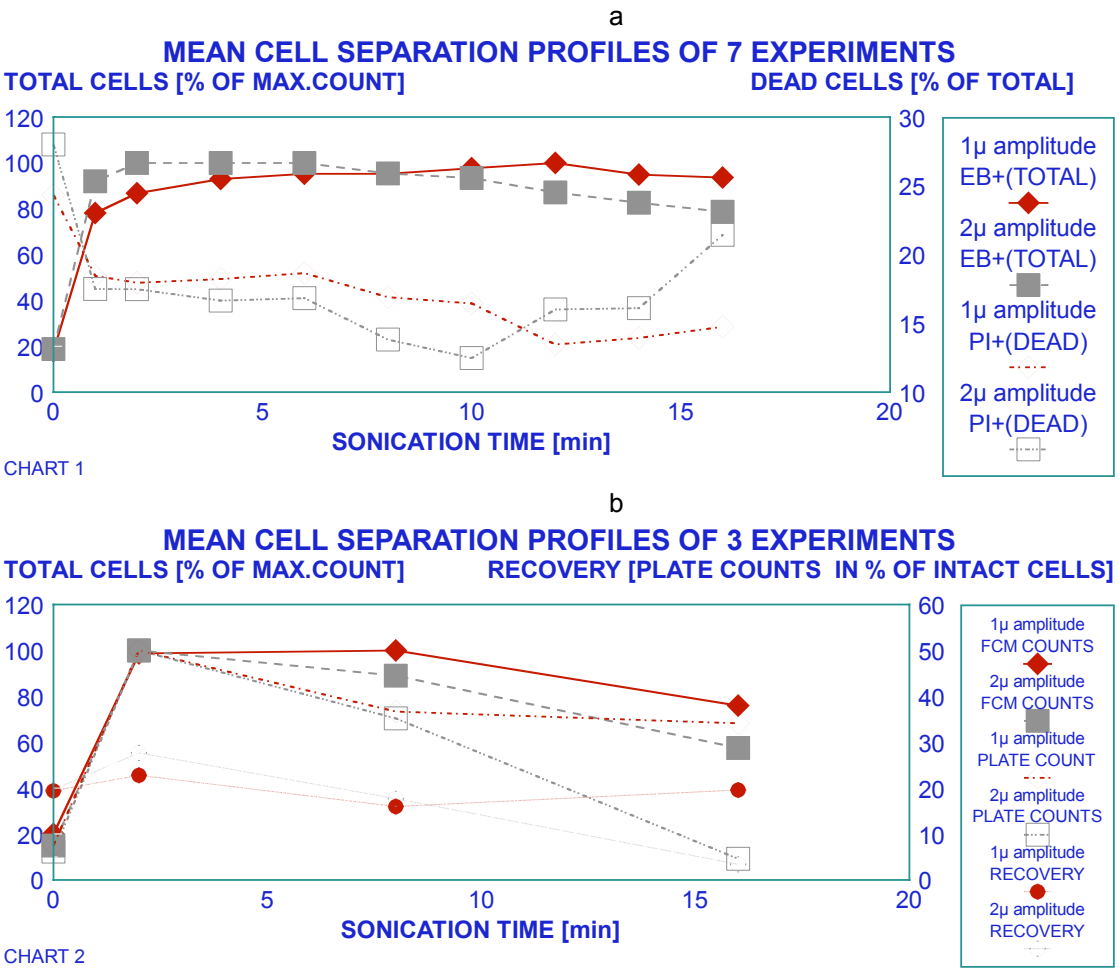


Figure 9

Effect of disaggregation on the recovery and enumeration of bacteria from dental plaque: The 5 fold increase in FCM counts of over the 2 minute sonication period (a) coincides with the highest recovery in graph (b). The decrease in the relative proportion of PI positive cells in (a) is a consequence of separating aggregates as described in the text. The loss of cell numbers at eight minute sonication at 2µm amplitude is apparent on both graphs.