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Flow cytometry of bacteria: glimpses from the past with a view to the future

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Abstract

Measurement of bacteria and other microorganisms at the level of single cells has progressed enormously over the last couple of decades. Up to the late 1970s, there were no other means than microscopy for observation of single microorganisms, making any type of measurement very cumbersome and tedious, at best. Today, we measure several parameters simultaneously with a precision of a few per cent, and at a rate of 1000 cells per second. The first papers on the use of flow cytometry to measure bacteria appeared only in 1977, although the method had proved highly successful in studies of mammalian cells for almost a decade. There were several reasons for this relatively late introduction, including technical limitations, problems with adequate staining, and, not least, the human factor. Today, flow cytometry has a wide range of microbiological applications, ranging from studies of the bacterial cell cycle and many other cellular characteristics to assessment of antibiotic susceptibility of clinical samples, and monitoring of bacteria and other microorganisms in anything from sewage to sea water. Still, the potential of flow cytometry in microbiology is far from fully utilised. Better instruments and new stains will provide new opportunities to understand, control and exploit this vital part of the biosphere.

1. When it all started

Flow cytometry as we know it today was developed during the last half of the 1960s. The first commercial instruments, the Cytofluorograph, a laser based instrument designed by Lou Kamentsky, and the Phywe ICP, which employed arc lamp excitation and was designed by Dietrich and Gøhde, both came out in 1970, and were immediately taken into use by immunologists and cell biologists.

Microbiologists took much longer to realize what a fantastic tool they had at hand. The first ones that I am aware of are Paau et al. (1977) and Bailey et al. (1977), both of whom published about 3 years after Becton Dickinson came out with its first FACS (Fluorescence-activated cell sorter). Another pioneer was Hutter, who published a series of flow studies of bacteria and various other microorganisms in the late 1970s (Hutter & Eipel, 1978; Hutter & Eipel, 1979a; Hutter & Eipel, 1979b).

Bailey et al. (1977) stained nucleic acid in *Bacillus subtilis* with propidium iodide (PI) and protein with fluorescein isothiocyanate (FITC) and saw that the amount of nucleic acid and protein per cell declined significantly as the culture approached stationary phase. They used a laser-based system and did not measure light scattering (LS). Paau et al. (1977), who used the same stains and also recorded

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light scattering, examined other bacterial species, with the same general conclusions. A year later, Hutter and Eipel (1978) published similar data, and some years after that, we confirmed that this decline and a decrease in cell size, occur during exponential phase as well (Fig. 1) (Skarstad et al., 1983; Steen, 1990a).

But that was about it. No one seemed to realize that they had hit a scientific gold mine. In fact, it seems to me that, even now, the majority of bacteriologists are not aware of the possibilities that flow cytometry offers for acquiring knowledge about bacteria which is difficult, if not impossible, to obtain by other methods.

Let us look at some of the implications of the findings I have just mentioned. First, a standard method for monitoring the density of a bacterial culture is to measure optical density (OD), for example at 600 nm, and the cell doubling time is determined by plotting such data versus time. However, that method is based on the (tacit) assumption that the size distribution of the cells, or more accurately, the light scattering, remains constant, since the OD is the product of cell number and cell size as measured by light scattering. The above results show that that assumption does not hold true.

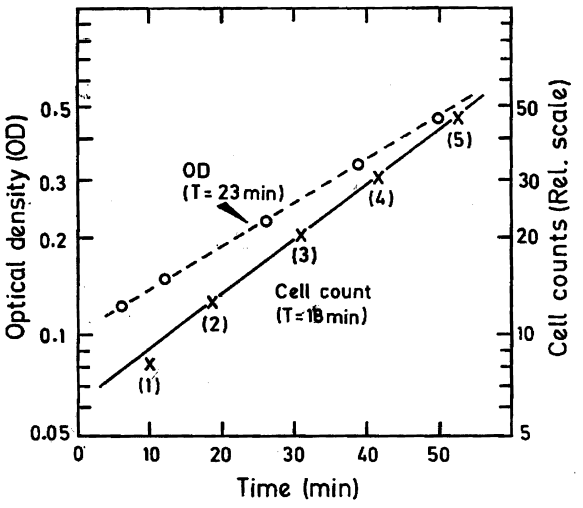

Fig. 1. Growth of a culture of *Escherichia coli* (in Luria broth) as measured by optical density (OD) at 600 nm (generation time calculated as 23 min) and by flow cytometric cell count (generation time calculated as 18 min) (from Steen (1990b), with permission of John Wiley & Sons, New York).

If cell size is decreasing as cell density increases, the cell number, as determined from OD, is underestimated. That also means that the doubling time calculated from OD measurements comes out longer than it should (Fig. 1).

Secondly, it means that when you pick a sample from an exponentially growing culture, you cannot assume that the cells are in the same state as cells picked from the same exponentially growing culture a little later, or that they are directly comparable to the cells picked from a similar culture by some other investigator. No wonder people doing studies of the bacterial cell cycle and related subjects, do not always arrive at the same conclusions.

Of even greater interest is the reason why the cells decrease in size. As is evident from Fig. 1, the decrease begins from the early log phase, that is, before any lack of nutrients has evolved, so, there must be another reason. It looks like the cells respond to cell density *per se*. In other words, cell density appears to be a parameter that somehow governs cell growth, which is to say that cells must somehow detect their numbers and communicate this in some way. We now know that they do, so the results of Bailey et al. (1977), Paau et al. (1977) and ours, for that matter, should have made bacteriologists jump in their chairs, first, because one of their standard methods was likely to produce misleading results, but second, and more importantly, because these results offered an obvious opportunity to make significant discoveries about bacteria.

So why didn't they take action? In fact, most of them still have not. It took quite some years before someone took flow cytometry and other methods into use to investigate these processes further. It turns out that bacteria are very social creatures indeed. They excrete factors that regulate a variety of physiological processes, including bioluminescence, antibiotic synthesis, virulence determinants, plasmid conjugate transfer, chromosome replication, and cell division (Withers & Nordstrøm, 1998). One obvious implication of these findings is that if bacteria can control each other, we should be able to control them using their own signals. If they can turn on and off each other's virulence or DNA-replication, it should be possible for us to do the same, once we have learned their language. Only now, some 20 years later, are we in the process of just that. Flow cytometry could

have accelerated this research substantially if it had been taken into use earlier and at its full potential.

There may be multiple reasons why it took so long for flow cytometry to catch on in bacteriology: One is that few if any bacteriologists knew or understood flow cytometry. Conversely, few of the people who were familiar with flow cytometry had any knowledge about bacteria and the problems bacteriologists were interested in. In any case, in the early days of flow cytometry, there were so many interesting problems to solve with mammalian cells, few investigators wanted to bother with bacteria, which are so much smaller, and therefore so much more difficult to measure. Another reason was that the flow cytometers commercially available at the time were not well suited for measurement of bacteria, partly due to lack of sensitivity, especially in light scattering, and partly because they did not have the right light sources to excite the appropriate dyes, in particular the highly DNA specific ones, like Hoechst 33258, DAPI, and mithramycin, which are excited in the UV or deep blue part of the spectrum. Ethidium bromide (EB) and propidium iodide (PI), which were used to stain DNA in mammalian cells (after treatment with RNase), also bind to RNA. Since bacteria may contain relatively much more RNA than do mammalian cells, and since RNase treatment of bacteria does not work as well as in mammalian cells, supposedly due to inhibition of the diffusion of the enzyme through the cell wall, DNA histograms of bacteria stained with EB and PI may be distorted by the fluorescence associated with RNA.

Flow cytometrists who ventured into the field may have been discouraged also by the absence of sharp peaks in their bacterial DNA histograms. In addition to what has already been mentioned with regard to inadequate instrument sensitivity and staining of RNA blurring the DNA-associated component of the histograms, there is a more basic reason for the unattractive histograms, namely, the very nature of the bacterial chromosome replication cycle. As long as the cells grow under near optimal conditions, new replication forks will commence before the chromosome is fully replicated, with the result that there is no part of the cell cycle in which the cell has a whole number of completed chromosomes, such as is the case during the G_1 and G_2 phases of the eukaryote cell cycle. Hence, the DNA histogram will lack the corresponding peaks. As is well known, flow cytometrists love to see distributions with sharp peaks, which prove that their instrument works properly and with sufficient sensitivity. I suspect that quite a few became discouraged and gave up when they obtained these broad DNA histograms. Finally, I think it is fair to say that many bacteriologists, and especially the ones working in clinical bacteriology at that time, were pretty conservative. They simply could not imagine anything better than heaps of petri dishes being processed by students, technicians, and other sources of slave labor.

2. A new and better instrument

I came into this field by chance, and I came into it not as someone with a bacteriological problem to solve, but as a physicist who likes to attack technical problems, especially if they can be solved by using light. One day I became fed up with waiting in line for the flow cytometer I used in a study of lymphocyte activation, and began thinking about how to make my own. Just a very simple one to measure only one fluorescence parameter, which was all I needed for those experiments. I realized that a flow cytometer is essentially a fluorescence microscope with cells flowing through the focus. That, of course, was not a new idea; the Phywe flow cytometer, which was a microscope-based instrument, had been in production since 1970. That I did not know, so I had to start from scratch. Having a fluorescence microscope on the bench, the main problem was to run the cells in a nice, narrow stream through the focus. The solution is shown in Fig. 2. Together with Tore Lindmo, I set up a spare nozzle from our laser-based flow cytometer, which he had built a few years earlier. We collected a photomultiplier tube (PMT) which I had used in some earlier experiments, some electronics, a multichannel analyzer, which is a device which accumulates data and presented them as histograms the way personal computer-based data acquisition systems now provided with flow cytometers do today, and put our instrument together in the course of a single day. In the evening that day we fixed and stained some thymus lymphocytes with mithramycin, and, before we went home, we had our first DNA histograms

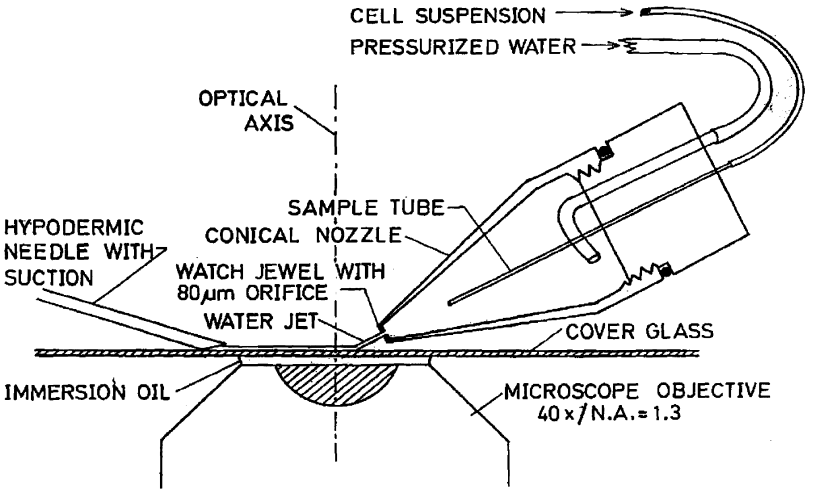

Fig. 2. An early version of the 'jet on open surface flow chamber' which facilitates measurement of fluorescence as well as light scattering in a flow cytometer with arc lamp epi-illumination through microscope optics (from Steen (1990a), with permission of John Wiley & Sons, New York).

(Steen & Lindmo, 1979). Probably the best lab day of my life. During the following days, we played further with our new toy, and soon we recorded fluorescence histograms with coefficients of variation (cv's) below 1%, and with that, our flow cytometer became an aim in itself.

Once I started using it, I realized that one fluorescence parameter was not sufficient, I needed light scattering. How to achieve that with the excitation light from the microscope objective coming out in a very wide cone, in contrast to the narrow laser beam, where light scattering can be measured just by situating the detector outside the beam? It took a while before I solved that problem, and before I could make it work, I had to pick apart the microscope objective and introduce between two of its lenses a circular piece of black paper, i.e., the field stop which gives rise to the dark field required to facilitate detection of scattered light (Fig. 3) (Steen, 1980). The device turned out to work so well that we could measure particles with diameters down to 0.2 μm. Later on we found a way to measure low and large angle scattering separately (Fig. 3) (Steen, 1986).

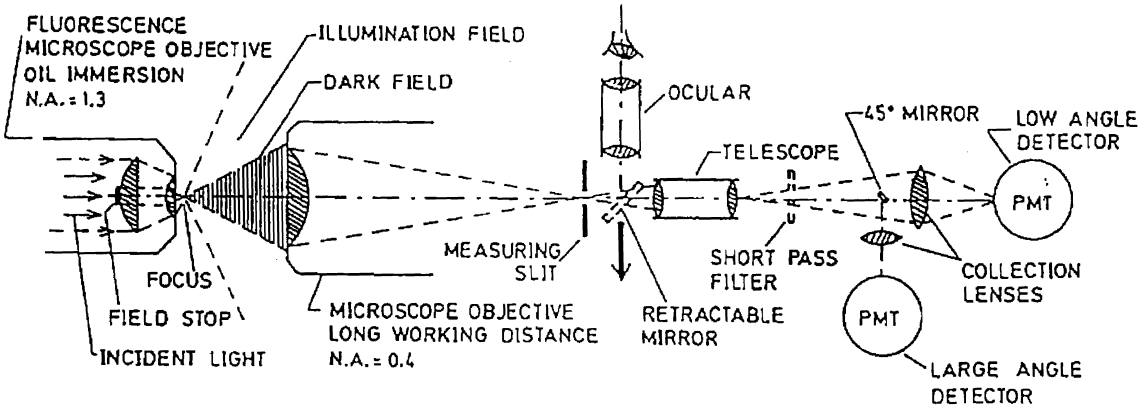

Fig. 3. Optical outline of the system used to detect light scattering at small and large scattering angles separately. The flow chamber of Fig. 2 is situated in the focus of the oil immersion microscope lens (from Steen (1990a), with permission of John Wiley & Sons, New York).

We were lucky enough to have a bacteriologist, Erik Boye, in the lab who was able to see the possibilities that flow cytometry offered. We ran some *E. coli*, and when he saw the featureless DNA histogram he declared the reason for the broad DNA peak possibly reflected some facts about the cell cycle of bacteria. To test this hypothesis he suggested we should try to grow *E. coli* with Rifampicin, and to our delight, we got histograms with nice, discrete peaks, which we interpreted as representing cells with an even number of chromosomes, mainly two and four, in accordance with what was known about the mode of action of this drug, namely that it inhibits RNA polymerase and thereby the initiation of chromosome replication (Fig. 4). That interpretation later proved correct.

Once it had been shown that it was possible to measure individual bacteria in flow, a lot of interesting applications were obvious, including cell cycle studies, counting and sizing of cells, measurement of physiological characteristics like membrane potential, clinical assays for antibiotic susceptibility, monitoring of fermentation processing, fresh water supply, and sewage processing. All of these applications have since not only proved feasible, but have yielded a lot of valuable knowledge as well.

Seeing the new possibilities that flow cytometry facilitated, Erik abandoned his research on DNA repair and went into the study of the regulation of the bacterial cell cycle, which he and his collaborators have pursued quite fruitfully since (Skarstad & Boye, 1994).

Since bacteria were so easy, I thought: why not try virus? So I tried some virus, admittedly some quite large ones, pox virus and T4 phage, to see what my light scattering device could manage, and with light scattering, virions could be detected (Fig. 5) (Steen, 1990a). With fluorescence, however, they could not, because I was not able to stain the DNA, supposedly because it proved impossible to hydrate these very dry particles. Again, I was not the first one to measure viruses. In 1979, Hercher et al. (1979) published a paper on virus detection using their custom built laser-based FCM. They measured T2 bacteriophage, which is fairly large, and could barely detect reovirus, which is 60×80 nm.

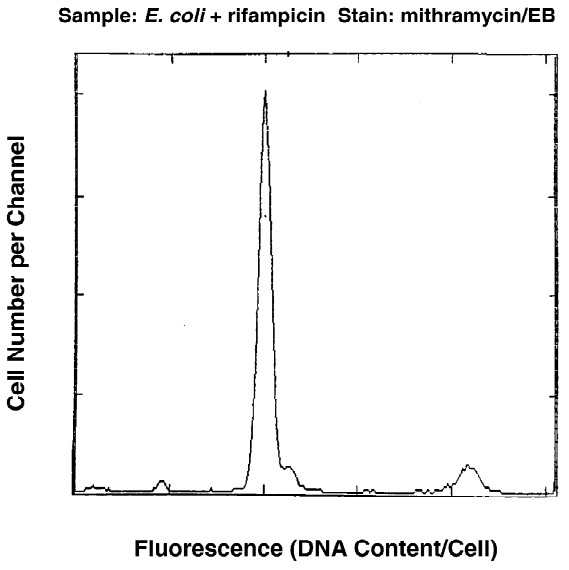

Fig. 4. Fluorescence histogram of *E. coli* grown in the presence of Rifampicin, fixed in 70% ethanol and stained with the DNA specific combination of mithramycin and ethidium bromide. The two prominent peaks represent cells with four and eight chromosomes.

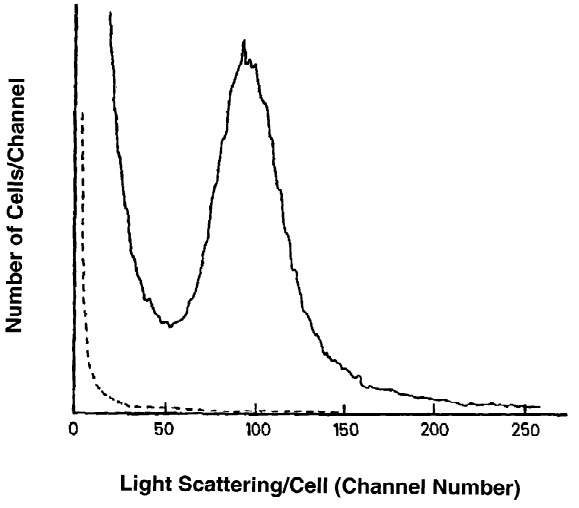

Fig. 5. Light scattering histogram of pox virus as obtained from a smallpox vaccine. The broken line is instrument background recorded with a sample of pure water, while the 'background' in the virus histogram is probably due to debris. The width of the histogram peak has a coefficient of variation CV=18%, which corresponds to a variation in the linear dimension of the virus of about 3%. This histogram was recorded with a microscope-based instrument (Fig. 3), but with a 1 W, 488 nm argon laser replacing the arc lamp (after Steen (1990b)).

The nice thing about detection of virus by light scattering is that it should discriminate very efficiently between different species on the basis of size. For particles in the size range of viruses, i.e., significantly smaller than the wavelength of light, the light scattering is proportional to the sixth power of the linear dimension. Hence, for example, viruses differing by only 5% in size should differ by about 30% in light scattering, and thereby be easily distinguishable. The problem is, however, if we can make it work for smaller virus. Probably not for the smallest. It can be calculated that the smallest particle that can be detected in light scattering is about 50 nm, assuming we have been able to increase the sensitivity of the FCM, as we know it today, to almost the theoretical limit. That leaves out quite a few types of virus. On the other hand, if we could stain their DNA properly we could detect even the smallest ones. Thus, it is feasible to measure DNA fragments containing only about 1000 base pairs (Goodwin et al., 1993). The problem is that while the DNA specific stains are all water-soluble dyes, the interior of a virus can be considered to be essentially dry. Nevertheless, Marie et al. (1999) have demonstrated staining of marine virus with SYBR Green-1 yielding sufficient fluorescence for flow cytometry.

Currently, detection of virus by light scattering is well below the sensitivity of any flow cytometer on the market. The fact is that there is probably no commercial instrument that can detect even the largest virus; most laser-based commercial flow cytometers have problems even with the light scattering signals from some bacteria. The reason for that is not lack of signal, but too much optical noise. That is, these instruments collect too much light coming from other sources than the cells or particles to be measured. This background light includes light scattered by the surfaces of the flow cell or the water jet of jet in air systems, by optical components, etc.

3. A few words about sensitivity

Sensitivity is crucial in flow cytometry of bacteria. Bacteria are about 1000 times smaller than the mammalian cells most flow cytometers were originally designed to measure, and they contain roughly that much smaller amounts of proteins and DNA. The sensitivity of flow cytometers depends on several factors. The popular notion is that the more light is used for illumination, the better the sensitivity, so the temptation is to use more powerful lasers. This is correct with regard to light scattering detection, the sensitivity of which increases by the square root of the light intensity. With fluorescence it is different, since during the passage through the focus of exciting light, each dye molecule may be excited many times, even by a laser emitting only a few tens of milliwatts, and there is a limit to how many excitations such molecules can stand. In other words, the dye is being bleached and loses its fluorescence as it goes through the focus, and that bleaching increases in proportion with the intensity of the excitation light, so with regard to fluorescence, one cannot buy oneself sensitivity just by installing larger lasers. Anyway, there is a very much cheaper way to increase excitation, namely, reducing the flow velocity instead of increasing laser power, since the slower the cell moves through the focus the more light it absorbs and the more fluorescence and scattered light it emits. However, that approach also aggravates the problem of dye bleaching. The extent of this depends on how easily the dye is degraded by light. Some dyes stand a lot of light, thousands of excitations, while others, like many porphyrins, are blown away by just a few. So what else can we do to improve sensitivity? We can reduce the background, or at least try to. The background is all the light coming from other sources than the cells: Raman scattered light from the sheath water, scattered light leaking through filters, fluorescence of optical components, and, in some cases, even ambient light from the room. It turns out (Steen, 1992) that:

$$\text{Sensitivity} \propto (\text{excitation intensity}/\text{background})^{1/2}$$

Hence, we can gain as much by halving background as by buying a laser twice as large, and that improvement costs nothing in terms of dye bleaching or measuring rate. The reason we use a pinhole, or field stop, in the image plane of the lens that collects the fluorescence in flow cytometers, is to suppress background light. There may be other paths to improvement as well, such as using better optical filters.

4. Antibiotic susceptibility

Since it turned out that our instrument could measure bacteria better than any other commercial instrument, we decided to pursue bacteriology. Clinical bacteriologists told us that if we could determine antibiotic susceptibility in hours rather than days, it could be very useful. From our experience with rifampicin, we knew that we could certainly detect very clear effects within 1 h or so. Thus, within that time, the histograms of both DNA content and cell size of exposed cells change dramatically, and, of course, we could keep track of the cell number. So we tested other types of antibiotics, and for all of them we saw easily detectable effects within the duration of a cell cycle (Walberg et al., 1997).

However, all of our initial work was done on cells fixed in ethanol; this was less than ideal because fixation takes time, and the wash steps involved cause cell loss. Hence, we decided to work on unfixed cells. That was not straightforward, because the dye did not penetrate the cell wall, unless we stained the cells on ice. Then the dye went in, but, as soon as we brought the cells back to room temperature, the fluorescence disappeared. We realized that bacteria may have a very efficient efflux pump, which pump out dyes, such as acridine orange (AO) and EB, against the concentration gradient (Jernaes & Steen, 1994). The next step was to apply metabolic inhibitors, like Na-azide and CCCP, and that almost solved the problem. What we saw was that a supposedly homogeneous culture of bacteria split up into distinctly separate sub-populations (Jernaes & Steen, 1994) (Fig. 6). Eventually, cells in the unstained population became stained, but when that happened, it happened quickly. We have seen this effect in many situations, but we do not understand why bacteria behave like that. Further studies of this phenomenon may reveal exciting new information about bacteria and how they interact.

Anyway, for the practical purpose of vital staining, we now knew how to do it, or so we thought. At least for *E. coli* we did, and we took it for granted that all bacteria, or at least all Gram-negative cells, would behave essentially the same way, but of course we had to check, and to our disappointment, we found that bacteria are more different than we had thought. Some species stain the way *E. coli* do,

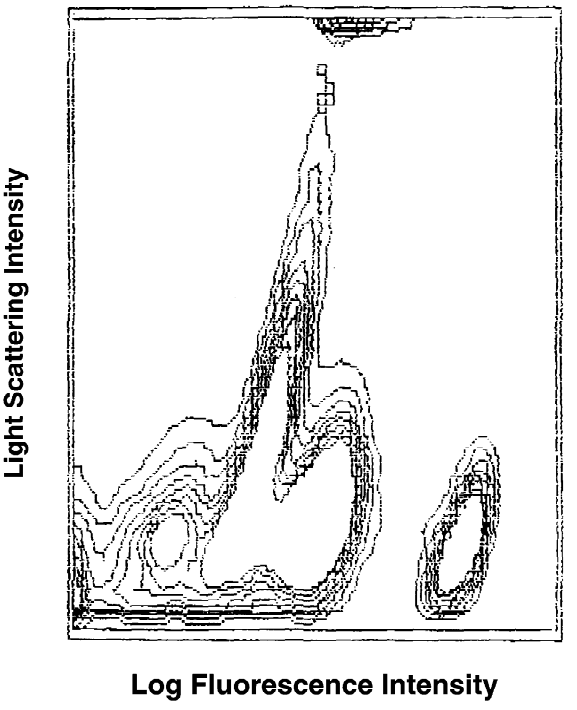

Fig. 6. Fluorescence/light scattering histogram of *E. coli* exposed to cold shock. The cells were put on ice while in growth medium for 20 min before being diluted in ice cold Tris buffer and subsequently stained with ethidium bromide. Note that the cells from a supposedly monoclonal and homogeneous culture split into four distincly separate subpopulations (after Jernaes & Steen (1994)).

others are quite different. Not only are they different with regard to one particular dye, but different dyes are also are taken up differently, even dyes which from a chemical point of view could be expected to behave similarly (Fig. 7) (Walberg et al., 1999). Frustrating, but true, and if there is one thing we should keep in mind as scientists, it is that the truth is never disappointing. To the extent that it is surprising it opens the possibility for new discoveries. In the present case, the fact that different bacteria react so differently to different dyes may conceivably be exploited for another purpose, namely as a means of identification.

Measuring DNA and light scattering is not the only way of testing antibiotic susceptibility. Another interesting approach is to measure membrane potential, which supposedly goes to zero when cells die (Kaprelyants & Kell, 1992; Novo et al., 1999). Flow

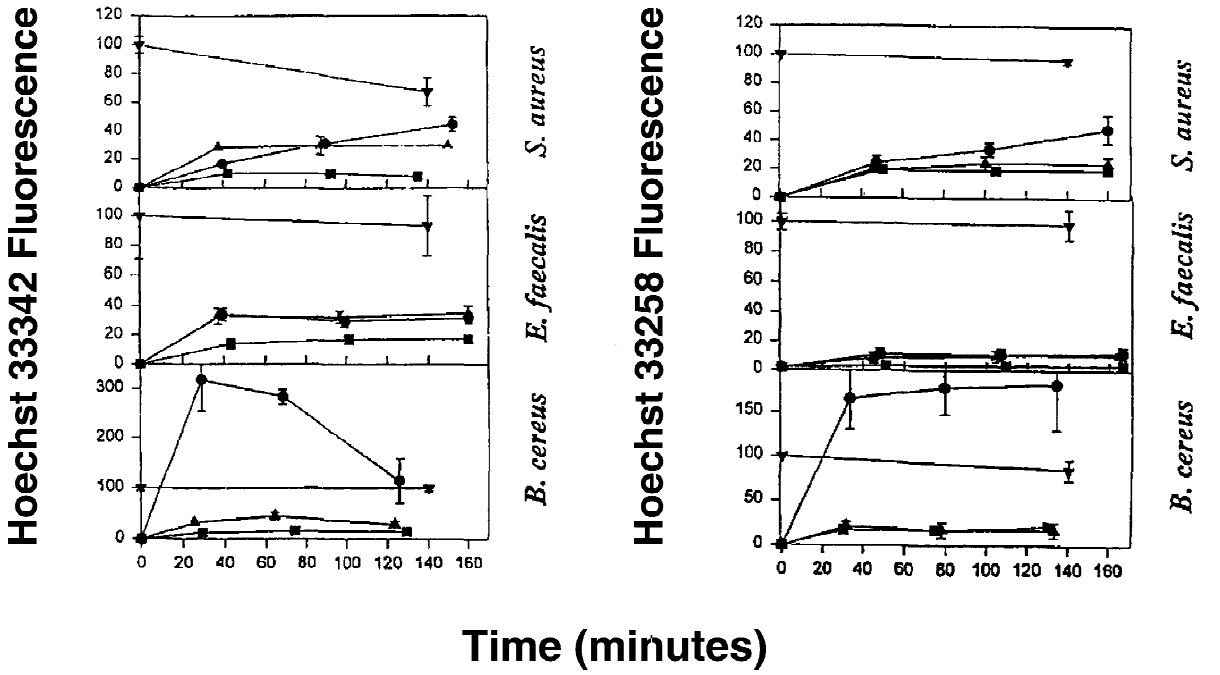

Fig. 7. Dye uptake of the DNA stains Hoechst33342 and Hoechst 33258 in three species of exponentially growing Gram-positive bacteria under various conditions. Inverted triangles: ethanol-fixed control cells. Upright triangles: unfixed cells at room temperature. Squares: unfixed cells on ice. Circles: unfixed cells plus metabolic inhibitor (Na azide) at room temperature. Note the significant differences in uptake between the two dyes, which are structurally very similar and bind to DNA in the same manner. Note also that for *B. cereus*, metabolically inhibited unfixed cells take up significantly more dye than cells which were fully permeabilized by means of ethanol fixation (after Walberg et al. (1999)).

cytometric antibiotic susceptibility has been assessed on this basis (Mason et al., 1994). So far, however, the principle has been applied to relatively few species. Furthermore, dormant bacteria may cause ambiguities whether one uses membrane potential sensitive dyes or measurement of DNA and light scattering (Kaprelyants et al., 1993).

Another problem in this regard is spores. Not only do they have no membrane potential, it is also very difficult to stain their DNA because they are so dry. The problem is essentially the same as with virus.

5. Current technology in flow cytometry

Most of the current technology in flow cytometry is essentially the same as 20 years ago.

5.1. Optics

Microscope optics are still the best. The use of optical fibers for illumination or light collection may save space, and increase ruggedness, but sacrifices performance in terms of sensitivity and resolution.

5.2. Fluidics

Nothing much has happened; commercial instruments still use sheath flow.

5.3. Electronics

These have become more integrated and densely packed, but that does not affect instrument performance, which was never really limited by the electronics anyway.

5.4. Detectors

Although avalanche diodes have been used in some instruments, photomultiplier tubes (PMTs) are still the detectors of choice. While some of these devices have been miniaturized, the performance is essentially the same as it was 20 years ago.

5.5. Light sources

The best lamps are still the 100 W high pressure Hg-, Xe-, and Hg/Xe-lamps used from the beginning of flow cytometry. We have got solid–state and diode lasers, especially the frequency-doubled, diode-pumped YAG (yttrium aluminum garnet) laser, emitting green light at 532 nm. A diode laser emitting in the violet at approximately 400 nm has just become available as a prototype (Nichia Corporation, Tokushima, Japan), and a solid–state laser at 473 nm is advertised by Capital City Lasers Ltd., Edinburgh, Scotland. While we are waiting for diode lasers at around 360 and 490 nm, we have to make do with the argon- and krypton gas lasers we have been using since the beginning.

5.6. Data acquisition and handling

One thing above all has really changed the game: the personal computer, with its processing capability and sufficient memory to store data in list mode, which facilitates gating, linear-to-logarithmic transformation, etc. in software.

5.7. Dyes

There are a lot of new dyes, which have greatly widened the range of applications of the flow cytometer. What we are still missing, however, is a truly DNA specific dye which can be excited with green, yellow or red light.

What we need is a flow cytometer which can measure small- and large-angle light scattering and fluorescence in three to four wavelength regions, with sufficient sensitivity for noiseless detection of bacteria. Dual-angle light scattering will facilitate distinction of cells on the basis of dry weight relative to volume and aid discrimination between live and dead cells. Furthermore, the instrument should have a light source which can excite the highly DNA-specific dyes, such as DAPI, Hoechst 33342 and 33528, and mithramycin, as well as the most important fluorescent dyes excited at higher wavelengths. For field studies, it is essential that the instrument be small, lightweight and sufficiently rugged. The technology for such an instrument already exists; it seems as while we, the market, are waiting for the industry to make it, the industry is waiting for the market to develop.

6. Conclusion

As demonstrated by this conference and several others, as well as by the steadily rising number of publications on bacteriological applications of flow cytometry, bacteriologists are increasingly becoming aware of the possibilities that this technology offers. Flow cytometry has had a very significant impact on mammalian cell biology, where it has numerous applications, and constantly is finding new ones as new problems arises and new methodological facilities become available — especially new dyes. There is no reason to believe that, with time, its impact on microbiology, and bacteriology in particular will be any smaller. On the contrary, there are so many species, each with so many strains, to investigate. There is such an enormous variation in the response of bacteria to growth conditions etc., and they differ so much in stainability, that bacteriology potentially represents a much larger area of application of flow cytometry than mammalian cell biology. In fact both fields are endless.

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