A PORTABLE FLOW CYTOMETER FOR THE DETECTION AND IDENTIFICATION OF MICROORGANISMS

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Flow cytometry can be used for the detection and identification of microorganism [1]. However traditional instrumentation is bulky and complicated to operate and align. These factors combine to make flow cytometry less than ideal for the field detection of biological agents.

Recently a new flow cytometer, the Microcyte [2], has been developed. The Microcyte is a battery-operated, portable instrument that is simple to use and needs no operator alignment and thus is well suited to field use. The Microcyte uses a 635 nm laser diode as the light source and we present data for a number of microorganisms stained with a variety of fluorescent stains amenable to excitation at this wavelength.

1. Introduction

Cytometry refers to the measurement (-metry) of cells (cyto-). These measurements may be of the cell's physical properties (length, volume, etc.) or of its biochemical properties (protein content, lipid content etc). Traditionally these measurements have been made by light or fluorescence microscopy. Microscopy is a labor-intensive task, prone to error and operator fatigue. It is also slow to perform and consequently conclusions are drawn from measurements of, at best, a few hundred cells. An alternative method of measuring protein content for example, would be to perform bulk biochemical measurements on a population of cells. However in doing this one makes the assumption that all of the cells in a population are behaving in a similar manner. The extent to which this assumption is true will vary with the cell type under investigation but in many cases the heterogeneity of a population of cells will be large, not least because of changes that occur as the cells progress *through* the cell cycle [3]. When samples are collected from the environment a variety of organisms and other particles will be present, increasing the heterogeneity still further.

The termflow *cytometry* refers to these same physical and biochemical measurements [4], but the measurements are made on *individual* cells as they flow past an array of detectors. The cell sample is introduced into the center of a stream of sheath fluid. The

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sheath fluid is pumped much more quickly than the sample and so the cells are constrained to the center of the sheath fluid. This process, known as hydrodynamic focusing, allows the cells to be delivered reproducibly to the center of the measuring point.

At the measuring point in a typical microbial flow cytometer [4-6], individual particles pass through an illumination zone, typically at a rate of some 1000 cells.s^{-1}, and appropriate detectors, which may be gated electronically, measure the magnitude of a pulse representing the amount of light scattered. The magnitudes of these pulses are sorted electronically into "channels", permitting the display of histograms of the number of cells possessing a certain quantitative property vs. channel number. Although many of the purposes for which one might use flow cytometry, such as microbial discrimination, require only a qualitative output, the essential character of the flow cytometric approach is strictly quantitative in nature.

The angular-dependence of scattered light provides further information on the nature of the scattering particles [7] but more importantly, appropriate fluorophores may be added to the cell suspension. These may be stains which bind to (or react with) particular molecules such as DNA, RNA or protein [8], fluorogenic substrates which reveal distributions in enzymatic activity [9], indicators which change their property as a function of internal pH [10] or which are taken up in response to membrane energization or, increasingly, antibodies [12] or oligonucleotidcs [13]tagged with a fluorescent probe. Flow cytometry thus represents a powerful method for the rapid analysis of heterogeneous microbial populations [3, 14, I5].

However, flow cytometry has historically possessed four major disadvantages that have greatly decreased its utility for the routine analysis of microbial cell suspensions. The first is the substantial cost: a typical laser-based flow cytometer capable of analyzing but not sorting might work out at *£75,000.* The second disadvantage is that because these are sophisticated instruments, skilled operators are usually required in order to obtain the optimum (and sometimes even any kind of acceptable) performance. Thirdly, in many models the sample flow rate is variable and not measured, so no *absolute* counts are possible. Fourthly, the majority of commercial instruments have been designed primarily for the analysis of animal (particularly human blood) cells and consequently bacteria are on the limit of detection [16, 17]. When one is considering instrumentation for the field detection of biological agents one must also be aware of other disadvantages such as the size (typically 1 to *5* m^3), lack of robustness for use outside of a laboratory, large power requirements (especially in laser-based models) and the fact that aerosols of the sample are produced in the analysis chambers of some instruments [18-20].

Recently a new flow cytometer [2] - the Microcyte - has been developed by M/s Optoflow A'S (Oslo, Norway), and commercialized by Aber Instruments Ltd, Science Park, Ceffi Llan, Abetystwyth, Wales, U.K. The Microcyte has been designed primarily for the analysis of microorganisms and detects light scattering and fluorescence from particles as small as 0.5 μm diameter in the standard configuration (and this can be adjusted to allow the detection of even smaller particles if required). The instrument is

small with dimensions of 33 x 43 x 16 cm and a weight ~12 kg. The light source is a low-power 635 nm laser diode allowing the instrument to be powered from internal batteries. In addition, the instrument is factory aligned and so does not require highly-trained operators. The sample flow rate in the Microcyte is carefully controlled via feedback, and so the instrument can be used to provide an accurate particle count for concentrations between $1x10^3$ and $1x10^7$ cells.ml^{-1} with an analysis time of less than 10 seconds. The sample analysis area is enclosed and no aerosols are generated during analysis, and after analysis bacteria are trapped in a disposable 0.2 μm filter.

Because almost all existing flow cytometers exploit the argon ion laser line exciting at 488 nm, relatively few studies have been carried out with 635 nm-excitable dyes. However, for most cell types (the main exception being plant cells) the autofluorescence background is significantly lower as one moves to the red end of the spectrum. However there are a growing number of suitable stains for this wavelength and data for some of these are presented.

2. Methods and Methods

Microorganisms

Bacillus subt His var niger spores: Spores from a dry preparation (CBDE, Porton Down, U.K.) were resuspended in K-HEPES buffer (see below).

Escherichia coil: (Gram-negative rod) *E. coli* was grown on L-broth (1% tryptone, 1% yeast extract and 70 $mg.l^{-1}$ $MgSO_4$, pH 6.8) at 37°C for 24 hours. The flask was then left unstirred at room temperature for 24 hours.

Micrococcus luteus (NCIMB 13267): (Gram-positive coccus) *M luteus* was grown on E-broth for 24 hours. The flask was then left unstirred at room temperature for 24 hours.

Baker's Yeast: An isolated strain was grown on YPG medium (5% glucose, *0.5%* yeast extract and 0.5% bacteriological peptone, pH *5)* at 30°C for 3 days.

Au media components were obtained from BDH or LabM.

Buffers

A K-HEPES buffer was used to dilute and/or resuspend cell samples for flow cytometric analyses. The same buffer was also used as a sheath fluid in the Coulter Epics Elite. The buffer contained 150 mM KC1 and 10mM HEPES. The pH of the buffer was adjusted to 6.8 before filtering through a 0.1 μm Whatman WCN filter.

The sheath fluid for the Microcyte was prepared in accordance with the manufacturer's manual and contained 0.1 mM sodium azide to inhibit microbial growth. The pH of the sheath fluid was adjusted to 3.5 by the addition of sulphuric acid and filtered in the same way as the HEPES buffer described above.

Sample preparation

All cultured microorganisms were diluted in K-HEPES to give appropriate concentrations for flow cytometric analyses (~$1x10^6$ $.ml^{-1}$). These organisms thus provided samples of high (but not 100%) viability. Dead samples were prepared by the addition of 70% ethanol to the above samples. After fixing for ~1 minute the fixative was removed by centrifugation and the samples were resuspended in sheath fluid.

Flow Cytometers

All samples were run on the Microcyte flow cytometer in accordance with the manufacturer's manual. In addition, all stained samples were analyzed on a Coulter Epics Elite flow cytometer using a 633 nm HeNe laser for excitation.

Particle Count

Samples containing a range of numbers of particles between 6 x 10^3 and 4 x 10^6 were prepared by dilution from a stationary phase culture of Baker's yeast. Particle counts were performed using the Microcyte in accordance with the manufacturers manual. A region of interest was set around the sample peak on the light scatter display and the analysis time was set to 2 seconds (1 µl). The sample concentration (in $particles.ml^{-1}$) was read directly from the integral display.

The particle concentration was also determined using a haemocytometer. Sample concentrations of greater than 10^6 $particles.ml^{-1}$ are required for accurate counts by this method and so only the highest two concentrations could be enumerated directly by this method. The concentrations of particles in samples with lower concentrations were determined from their dilution factors.

Fluorescent Stains

The following dyes were obtained from Molecular Probes Ltd, P0 Box 22010, Eugene, Oregon 97402, USA [21].

TO-PRO-3 was obtained as a 1 mM solution in DMSO. It was added to samples to give a final concentration of 1 µM.

Oxonol V was dissolved in ethanol at a concentration of 1 $mg.ml^{-1}$. This was added to samples to give a final concentration of 1 $\mu g.ml^{-1}$.

3,3'-diethyl-thiadicarbo-cyanine iodide ($DiSC_2(5)$) was dissolved in filtered, distilled water at a concentration of 1 $mg.ml^{-1}$. This was added to samples to give a final concentration of 1 $\mu g.ml^{-1}$.

SYTO-17 was obtained as a 1 mM solution in DMSO. It was added to samples to give a final concentration of 1 µM.

3. Results and Discussion

There are many instances in microbiology where a reliable count of the number of particles present in a sample is required. This is true in an industrial or research setting where one may wish to monitor the progress of a fermentation. In the case of bio-warfare agent detection the particle count can be used to give an indication of bacterial load in an air sample and may serve as a useful trigger for other more specific tests. Figure 1 shows a comparison of counts of yeast performed using the Microcyte and by haemocytometry. Only the two most concentrated samples could be enumerated by haemocytometry. In this case haemocytometry took 10-15 minutes per replicate, while 3 counts were done on the Microcyte in less than 30 seconds.

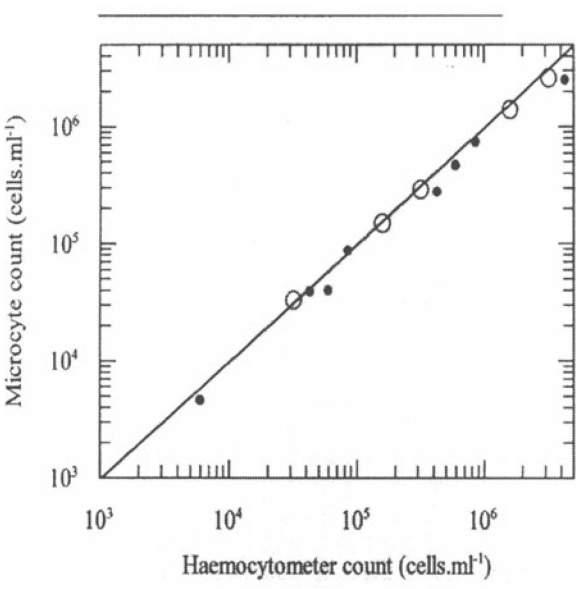

Figure 1 : Comparison of haemocytometer and Microcyte counts. • = Yeast, O = 5 μm latex beads. The best fit line by linear regression has a slope of 0.970, an intercept of 0.079 and a correlation coefficient of 0.996.

Fluorescent stains such as TO-PRO-3 which are excluded by the membranes of intact cells can be used as the basis of flow cytometric viability measurements. Figure 2 shows the results of such an assay, performed with Baker's yeast. Two separable populations can clearly be seen in the stained, unfixed sample; according to the dye exclusion method of viability determination those with lower fluorescence are "viable", while those that are more brightly fluorescent are dead.

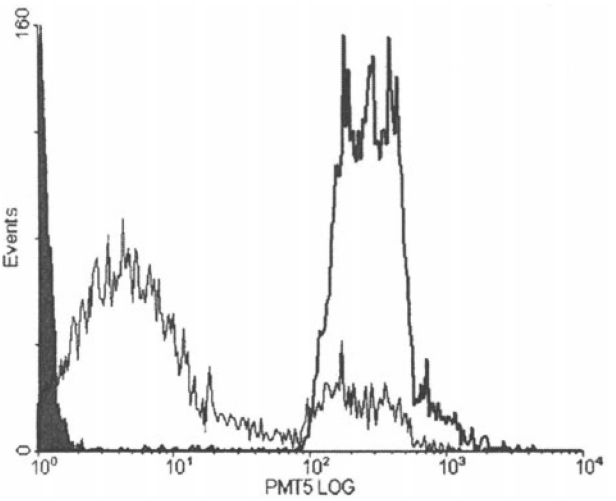

Figure 2 : Flow cytometric analysis of Baker's yeast performed on the Coulter Epics Elite. The 633 nm HeNe laser was used for excitation and fluorescence was collected via a 675 nm band-pass filter. The amplifier gain was logarithmic. ■ Unstained yeast, thin line = unfixed yeast, thick line = fixed yeast. TO-PRO-3 is excluded by the membrane of viable cells but freely enters dead (fixed) cells. In the example shown 18.7% of the unfixed yeast suspension were brightly stained and according to this method would be recorded as dead.

Some fluorescent stains also offer a level of discrimination between microorganisms. Figure 3 shows the results of flow cytometric analysis of a range of microorganisms stained with a variety of fluorescent stains. The data are displayed as collected on the Coulter Epics Elite. However, the results obtained on the Microcyte were qualitatively the same.

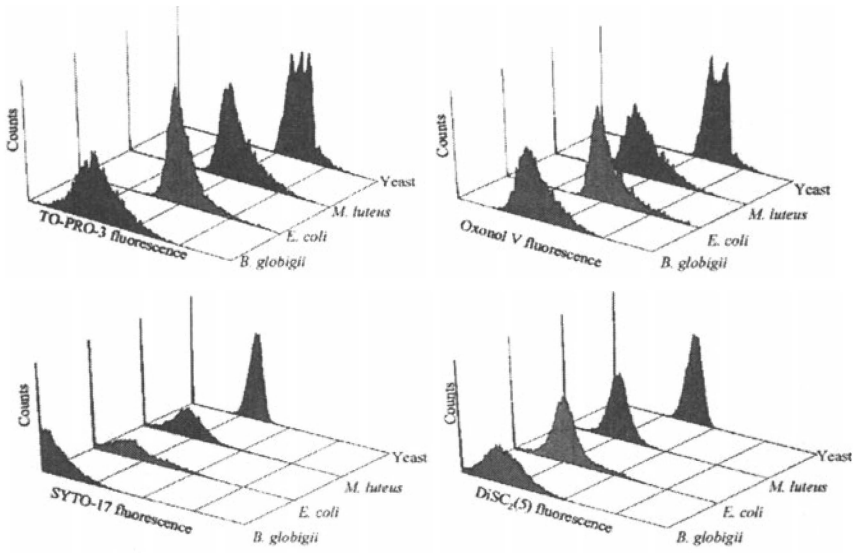

Figure 3: Flow cytometric analysis of *B. globigii*, *E. coli*, *M. luteus*, and yeast with four red-excitable fluorescent stains. The data were collected using the Coulter Epics Elite flow cytometer as described in the legend to Figure 2. In most cases the spores of *Bacillus globigii* are somewhat less fluorescent than the vegetative cells of the other organisms measured.

Figure 4 shows the broad distribution of light scattering properties exhibited by *Bacillus globigii* spores. This is in contrast to the tight distribution observed in 1 μm beads. This is due to the fact that spores form clumps with each other and with cell debris [22].

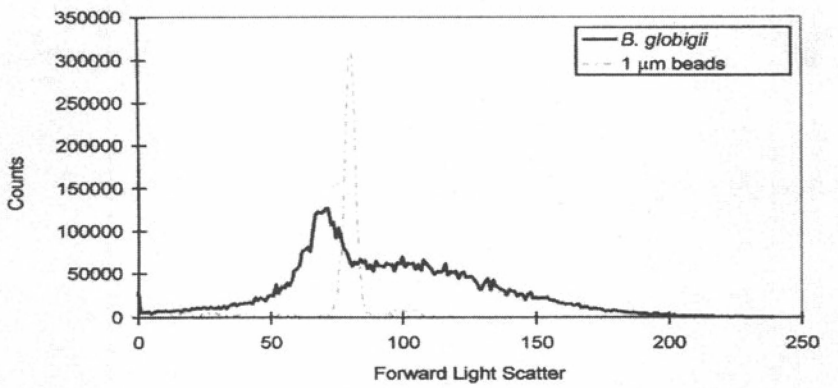

Figure 4: Flow cytometric analysis of *Bacillus globigii* was performed on the Microcyte as described in the materials and methods. The logarithmic amplifier was used for both samples. A broad distribution of light scattering from the spores is observed, however the tight distribution observed for the 1 μm diameter beads shows that the Microcyte is functioning optimally.

In conclusion, flow cytometry has many advantages for the detection of biowarfare agents, not least because of the fact that single cells are analyzed and consequently identification of these agents against a background of other particulates is possible. New instrumentation is beginning to overcome many of the historical disadvantages of the technique and we can expect this to continue in the future. There is a constantly expanding range of fluorescent dyes and these encompass a wide variety of excitation wavelengths. Many of these offer some level of discrimination between particle types. Where the presence of a particular organism is suspected fluorescently labeled antibodies will offer a higher level of specificity, but initial experiments with broad-specificity dyes will continue to be an important first step in the detection and identification of microorganisms.

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