Flow cytometry (FCM) is a rapid technique for the analysis of individual cells. Light scattering and fluorescence properties of cells are analysed as the cells pass through a laser beam, and, in specialised instruments, cells with specific characteristics can be isolated. This review article describes FCM and discusses recent advances that may be expected to increase its use in clinical microbiology. New applications include susceptibility testing, where FCM allows death or damage to microorganisms to be identified without the necessity to observe microbial growth, as well as monitoring the status and extent of infection in HIV-positive patients.

Flow cytometry (FCM) has many advantages over conventional cytometry. Firstly, since acquisition rates of up to 10,000 cells sec$^{-1}$ can be achieved (depending on the instrument used), flow cytometric data sets often represent measurements of in excess of 100,000 cells. In contrast, measurements by microscopy often involve only a few hundred cells. The increased sample throughput of FCM leads to the acquisition of statistically significant results and the detection of rare cell types. Secondly, since FCM uses very sensitive electronic detectors to measure the intensity of scattered light or fluorescence at a given wavelength, different intensities of light scatter/fluorescence can be distinguished. By calibrating an instrument with samples of known size or fluorescent intensity, it is possible to obtain quantitative measurements. Thirdly, by using dichroic filters to optically separate light of different wavelength, flow cytometric measurements can be made on several different characteristics of each cell. Typical commercial flow cytometers allow 5–10 different parameters (e.g. size, protein content, DNA content, lipid content, antigenic properties, enzyme activity, etc.) to be collected for each cell, allowing the operator to distinguish between different cell types. Finally, since measurements are made on single cells, heterogeneity within the population can be detected and quantified in a way that cannot be achieved by other means.

Whilst all commercial flow cytometers have the advantages described above, some specialised instruments (cell sorters) are able to physically separate cells on the basis of user-defined characteristics. Depending on the instrument, cells may be bulk-sorted or individual cells may be sorted onto microscope slides or microtitre/agar plates. Providing that appropriate cell staining and sample preparation methods have been used to maintain viability, sorted cells can be grown to give clonal colonies or broth suspensions for confirmation of identity via standard clinical microbiology methods.

Over recent years a number of reviews of FCM have been published [see examples in reference 1]. The purpose of this review is to highlight the value of FCM for clinical samples, with particular reference to microorganisms.

**Clinical applications of microbial detection by FCM**

The detection of bacteria or yeasts in body fluids is important for the diagnosis of a number of different diseases. Urine may contain a variety of particulates, including red and white blood cells, epithelial cells, bacteria and inorganic chemical crystals. The presence and concentrations of these particulates can be used for the diagnosis of a range of diseases and disorders. Flow cytometers designed specifically for urinalysis are available commercially and can allow the simultaneous determination of many different cell types [2]. These devices have been shown to be more sensitive than manual microscopic methods [3].

In comparison to the relatively straightforward detection of bacteria in urine samples, blood is a much more challenging sample type to use. In clinical infections such as bacteraemia, concentrations of the contaminants may be on the order of 10 bacteria in 1 mL of blood, whilst the number of red blood cells is $>10^9$ per mL. The high ‘background’ cellular load of blood makes the detection of bacteria by microscopic methods all but impossible. Consequently, although bacteraemia is a potentially life-threatening condition, diagnosis relies in many cases upon the growth of bacteria in media inoculated with samples of whole blood. However, methods are available to selectively lyse the erythrocytes in a blood sample, leaving a sufficiently low cell concentration to allow the rapid sample throughput capabilities of the flow cytometer to be utilised for the detection of bacteria. A number of products are now available commercially to achieve this, for example, CyLyse from Partec GmbH, Münster, Germany.

Mansour and colleagues [4] developed a model system in which they used ethidium bromide labelling to nonspecifically detect *Escherichia coli* in blood at concentrations of 10 - 100 cells.mL$^{-1}$. The sensitivity was 100 to 1000-fold better than that achieved using microscopy techniques, and took just 2 hours to perform, including sample preparation. In clinical presentations where bacterial concentrations are less than 10 per mL, a short pre-incubation step prior to flow cytometric analysis may be envisaged to increase the bacterial load of the sample to a level where it may be detected.

The detection of specific pathogenic microorganisms in clinical samples has been much improved by the availability of monoclonal antibodies. These antibodies can be fluorescently labelled (either directly or indirectly) to enable them to be detected flow cytometrically. A variety of fluorescent labels are available, the most common is fluorescein isothiocyanate (FITC). This has the advantage of being well-excited by the 488 nm Argon ion laser which is used as standard in most flow cytometers. Other (spectrally-distinct) molecules such as allophycocyanin, Texas Red and phycoerythrin allow multiple targets to be detected simultaneously. The labelled-antibody approach has proven to be useful for the detection of mycobacterial species from clinical (sputum) specimens [5]. Yi and colleagues showed that Mycobacteria could be detected...
Susceptibility testing

In an era of worrying and increasing levels of antibiotic-resistant pathogens, it is not surprising that understanding the interactions between microorganisms and the drugs designed to kill them has become another important area for the clinical application of flow cytometric methods. A variety of fluorescent stains for assessing the viability of microorganisms have been identified [Table 1, see also reference 6] and these are particularly useful for determining the efficacy of antimicrobial compounds. Microorganisms exposed to antibiotic or antifungal compounds (either in vivo or in vitro) are compared to control (untreated) samples and appropriate stains are used to identify changes in nucleic acids, proteins, membranes, etc.

Antibiotics disrupt cellular activities and the particular mode of action can be determined flow cytometrically. For example, antibiotic-induced damage to cell membranes can be detected by the entry of fluorescent compounds (such as propidium iodide) which are normally excluded by the intact cell membrane. Alternatively, to determine the response of cells to an antibiotic, which affects nucleic acid synthesis, one could use a stain such as DAPI, which binds to DNA, or pyronin Y, which binds to RNA.

In addition, FCM permits subpopulations with varying resistance to be identified and accurate assessment of the dose-response curve can also be performed as part of the assay [see examples in reference 7]. Flow cytometric susceptibility testing thus allows death or damage of microorganisms to be identified without the necessity to observe microbial growth (or lack thereof). Flow cytometric susceptibility testing can be performed in a few hours [Figure 2] and consequently this method has the potential to contribute to the decision of which drug or drug combination would be most appropriate for a particular patient.

HIV

FCM has been used to great effect for monitoring the status and extent of HIV infection. Whilst viral antigens can be detected by FCM [8], monitoring of HIV infection usually relies on regular quantitation of lymphocyte populations. The absolute numbers of CD4+ lymphocytes and their percentage values within the total lymphocyte populations are good indicators of the disease and its progression. Fluorescently-labelled antibodies can be used to selectively label different types of lymphocytes and thus FCM has an important role to play not only in disease surveillance, but also in determining the efficacy of treatment. Ideally analysis of blood samples should be performed within hours of collection. Unfortunately, the majority of HIV-infected individuals are not within easy reach of the specialised laboratories capable of performing these tests. A mobile flow cytometry laboratory has recently been developed to address this issue (Partec GmbH, Münster, Germany). The CyFlow flow cytometer is installed in an off-road 4-wheel drive car and is powered using 12 V DC car batteries charged by solar panels [Figure 3]. The system has advantages over many flow cytometers in that lymphocyte populations can be simultaneously identified and quantified without the addition of reference controls [9]. Detection of the different lymphocyte populations is achieved using monoclonal antibodies targeted against the appropriate CD markers. The cells in a fixed volume (200 mL) of sample are counted; counting is switched on and off using an electrode to sense the depth of fluid in the sample tube. The combined detection and counting not only simplifies the procedure, thus reducing the potential for error, but also minimises costs.

Table 1. Some fluorescent dyes used for determination of viability by FCM.

<table>
<thead>
<tr>
<th>Stain</th>
<th>Mode of Action</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>BacLight Kit: Molecular Probes – <a href="http://www.probes.com">www.probes.com</a></td>
<td>Propidium iodide excluded by intact membranes. All cells take up SYTO9</td>
<td>Live cells are green, dead cells are red.</td>
</tr>
<tr>
<td>bis(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC4(3))</td>
<td>Uptake by dead cells</td>
<td>Dead cells appear green/yellow.</td>
</tr>
<tr>
<td>Calcofluor White</td>
<td>Uptake by dead cells</td>
<td>Dead cells appear blue.</td>
</tr>
<tr>
<td>5-cyano-2,3-ditolyltetrazolium chloride (CTC)</td>
<td>Respiratory activity</td>
<td>Live cells appear red.</td>
</tr>
<tr>
<td>Fluorescein diacetate/ Carboxy-fluorescein diacetate</td>
<td>Enzymic activity</td>
<td>Live cells appear green.</td>
</tr>
<tr>
<td>Rhodamine 123</td>
<td>Uptake by live cells</td>
<td>Live cells appear green.</td>
</tr>
<tr>
<td>TO-PRO-3 / Propidium iodide</td>
<td>Excluded by intact cell membrane</td>
<td>Dead cells appear red.</td>
</tr>
</tbody>
</table>

Figure 2. Antimicrobial susceptibility testing using flow cytometry. Two colour fluorescence histograms of Enterococcus faecium treated with vancomycin and stained with the FAST-2 kit (BioRad). With increasing exposure time, an increase in the number of dead and dying cells (events present in quadrants 2, 3, and 4) was observed. Data collected by Kao-Ping Chiu and colleagues at BioRad, printed with permission (The Purdue Cytometry CD-ROM Volume 4, J. Watson, Guest Ed., J. Paul Robinson, Publisher. Purdue University Cytometry Laboratories, West Lafayette, IN, USA. 1997, ISBN 1-890473-03-0).

Figure 3. The CyFlow flow cytometer, image kindly provided by Partec, GmbH.
References
1. Davey HM, Kell DB. Flow cytometry and cell sorting of heterogeneous microbial populations—the importance of single-cell analyses. Microbiological reviews 1996;60(4):641-696.

The author
Hazel M. Davey, Ph.D.,
Postdoctoral Research Assistant,
Institute of Biological Sciences, University of Wales,
Aberystwyth, Ceredigion, SY23 3DJ,
Wales, U.K.
Tel.: +44 1970 621829
Fax: +44 1970 622307
Email: hml@aber.ac.uk
Website: http://qbab.aber.ac.uk/home.html