SKATGRAF: A Stand-alone Program for the Calibration and Plotting of Flow Cytometric Data

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*Flow cytometry is a rapid and powerful method for measuring the fluorescence and light-scattering properties of individual cells. The Skatron Argus instrument is a relatively low-cost flow cytometer developed for use with microbial cells. In common with other rapid methods large amounts of data are collected and thus an efficient method of data analysis is required. To this end we have developed **SkatGraf** to import the data files acquired by the software system provided with the Skatron Argus flow cytometer. **SkatGraf** permits the user to display and manipulate graphs of the data on the screen, to print the graphs and to convert channel numbers to absolute values of cell size or relative fluorescence as well as to export the data in formats suitable for use in other applications.*

Introduction

It is becoming widely appreciated that a macroscopic description of a microbiological culture, in terms of its DNA content, protein content, viability etc. is not sufficient to describe the culture fully (Kell *et al.*, 1991). This is because macroscopic measurements provide us only with an *average* value for the population and as such give us no measure of the *range* or *distribution* of the properties of interest within the population. Worse still, attempts to relate *macroscopic* measurements of biochemical properties to the physiological states of *individual* cells, including even whether they are alive, dormant or dead, can only be at best misleading (Davey *et al.*, 1993 *a*; Kaprelyants & Kell, 1992; Kaprelyants *et al.*, 1993) What is required is a rapid method of making these measurements on *individual* cells. The technique of flow cytometry makes this possible and is being increasingly applied in microbiology (Lloyd, 1993; Shapiro, 1990; Steen, 1990 *a*).

Flow cytometry enables one to make rapid measurements on particles or cells as they flow in single-file through a sensing point. Typical rates for these measurements are in excess of 1000 cells s^{-1} (Boye & Steen, 1993; Steen, 1990 *b*; Steen *et al.*, 1990).

In the Skatron Argus 100 flow cytometer the sample of interest is injected into the centre of a stream of rapidly moving sheath fluid and the cells pass over a cover slip where they interact with a beam of light from a high-pressure mercury arc lamp (Boye & Løbner-Olesen, 1990; Steen & Lindmo, 1985). As the cells pass through this measuring point appropriate detectors, that may be gated to exclude debris or certain cell subsets, measure the amount of light scattered or, via suitable filters, the fluorescence of the particle (Steen, 1990 *a*). The collected data are converted into electrical pulses and passed to a multichannel pulse height analyser where they are 'binned' according to their size into channels that increase in number with increasing levels of scattered light or fluorescence (Skarstad *et al.*, 1985; Steen *et al.*, 1982).

Channel numbers enable one to express the data in terms of the relative light scattering or fluorescence of

each cell, but for many purposes one would wish to describe the cell population in absolute terms. In order for this to be achieved a calibration method for converting the channel numbers into absolute values is required.

One of the more common applications of flow cytometry is the estimation of cell size. In a few suitably equipped flow cytometers cell size is determined by the analysis of pulse width (Sharpless *et al.*, 1975), but in most flow cytometers (including the Skatron) cell size measurements are achieved by measuring the extent of light scattered at low angles in a forward direction. (Boye *et al.*, 1983; Frelat *et al.*, 1989; Kerker, 1983; Pinder *et al.*, 1990; Salzman *et al.*, 1990; Shapiro, 1988; Steen *et al.*, 1989). However, it has been shown that there is a non-linear relationship between the extent of forward-angle light scattering and the size of the particle (Salzman *et al.*, 1979, 1990; Davey *et al.*, 1990), although this non-linearity is conveniently fitted using a 3-term polynomial. Further, even when a calibration with latex beads of known size is carried out a significant underestimation of the true cell size is obtained; to overcome this problem an additional calibration based on the difference in scattering properties of cells and latex beads may be used (Davey *et al.*, 1993 *b*). It should be noted that linearization and calibration of forward light scatter data for the estimation of cell size is only suitable for pure cultures of microorganisms. Such calibrations are not appropriate for mixed cultures where variations in refractive index may contribute substantially to observed differences in light scattering intensities (Shapiro, 1977).

In the work described, we found that available software was rather inflexible for performing the analyses which we required. In addition the Skatron data files do not conform *exactly* to the Flow Cytometry File Standard (Murphy & Chused, 1984) and so are not easily processed by commercial flow cytometry packages. We have therefore developed *SkatGraf* to enable flow cytometric data obtained with the Skatron Argus 100 to be graphically displayed and printed. *SkatGraf* also features a calibration option, allowing the user to enter calibration data,
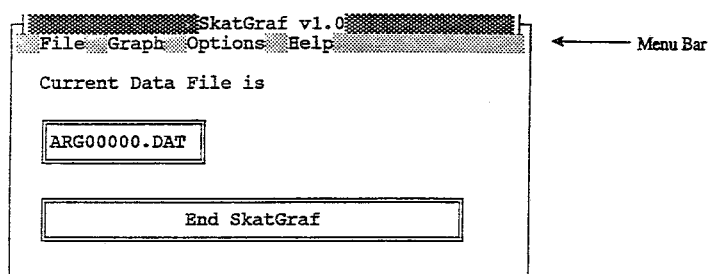
◄──── Menu Bar

Figure 1 The main *SkatGraf* window from which most commands are issued.

```
FCS 1.0S    58   929   932  33286    0    0_
$SYS_SKATRON MS-DOS_
$INST_SKATRON LAB_
$CYT_SKATRON ARGUS_
$FIL_\SKATRON\HAZE\9107\ARG26001.DAT_
$ASC_T_$DATE_26-JUL-91_$BTIM_10:08.12_$ETIM_10:08.54_
$SMNA_calibration beads        _
$SHFS_ 1.71_
$SAFS_ 50.0_
$TOT_21126_
$MODE_U_$WIND_P1:1,254_
$P1B_*_$P1R_256_
$P1N_LS1_$P1V_500_$P1G_LOG_$P1I_2_$TR_LS1,10_
$MODE_U_$WIND_P1:1,254_
$P2B_*_$P2R_256_
$P2N_LS2_$P2V_700_$P2G_LOG_$P2I_2_
$MODE_U_$WIND_P1:1,254_
$P3B_*_$P3R_256_
$P3N_FL1_$P3V_900_$P3G_LOG_$P3I_2_
$MODE_U_
$P4B_*_$P4R_256_
$P4N_FL2_$P4V_300_$P4G_1.00_$P4I_2_
$MODE_U_
$P5B_*_$P5R_256_
$P5N_FL3_$P5V_300_$P5G_1.00_$P5I_2_
$MODE_C_$WIND_P1:1,254_
$P1B_*_$P1R_64_$P2B_*_$P2R_64_
$P1W_1,254_$P2W_1,254_
$MODE_C_$WIND_P1:1,254_
$P1B_*_$P1R_64_$P3B_*_$P3R_64_
$P1W_1,254_$P3W_1,254_
$MODE_C_$WIND_P1:1,254_
$P2B_*_$P2R_64_$P3B_*_$P3R_64_
$P2W_1,254_$P3W_1,254_
```

Figure 2 The text area at the start of each Skatron file contains information about the contents of the file. *SkatGraf* reads the $SMNA field to determine the identity of the sample, in this case 'calibration beads'. The two lines following each $MODE_U field describe each single parameter histogram stored while the two lines following each $MODE_C field describe the dual parameter histograms stored.

such as bead size and channel number, or fluorescence intensity and channel number, along with an optional additional calibration factor in order to calibrate and linearize their data.

Materials and methods
Hardware
SkatGraf runs on IBM PCs and 100% compatible machines, but requires at least a 386 processor. Most screen adapters are supported, including Hercules, EGA and VGA and some SVGA monitors. Printers supported by the program include Postscript, HP LaserJet II and III, HP DeskJet, HPGL and Epson. In addition a coprocessor and mouse, although not required for use of *SkatGraf*, are supported, and where used enhance the speed and ease of use of the program, respectively.

Software
SkatGraf is written in Microsoft *Visual BASIC* for MS DOS Professional Edition Version 1.0. The graphs are produced by shelling to the *gnuplot* (version 3.2 for DOS 386) package which is in the public domain.

Flow Cytometry
Flow cytometry was carried out on a Skatron Argus 100 flow cytometer (Skatron Ltd., PO Box 34, Newmarket, Suffolk, UK), as described elsewhere (Boye & Løbner-Olesen, 1991; Davey *et al.*, 1993b; Steen & Lindmo, 1985; Allman *et al.*, 1991; Allman *et al.*, 1992; Edwards *et al.*, 1992). The flow cytometer was set up as described in the manual, save that an additional 0.1 µm filter was placed in the sheath fluid line. The sheath fluid was prepared from singly-distilled water that had been further purified using Millipore MilliQ apparatus. Sodium azide was added at a concentration of 1 mmol l^{-1} to inhibit microbial growth in the sheath fluid tank. Details of the optical characteristics of the filter blocks used are given in the legends to the appropriate figures.

The flow cytometer was controlled by a Viglen IIHDE microcomputer (IBM PC AT-compatible, 286 processor, EGA screen) with software supplied by Skatron. In the present work data were collected from the forward scatter detector (LS1), the wide-angle scatter detector (LS2) and from a fluorescence detector (FL1). In all cases the measurements were gated by the LS1 channel and the photomultiplier gains were logarithmic. The photo-multiplier (PMT) voltages used differed between samples and are given in the legends to the appropriate figures.

The program
An Overview of SkatGraf
SkatGraf is a mouse-aware, fully menu-driven program, written in Microsoft *Visual BASIC* for MS DOS. It is designed to enable the user to display flow cytometry data as either single or dual-parameter histograms and allows linearization and calibration of the data for the single parameter histograms. A full on-line help system is provided, describing all of the main features of the program along with a description of how to navigate the program with both the keyboard and the mouse. Although *SkatGraf* is a DOS-based package the graphical-user-interface should make its use intuitive to anyone familiar with Microsoft *Windows*. The program is started from the DOS command line by changing to the directory where *SkatGraf* is installed and issuing the command SKATGRAF. When the program has loaded, the main *SkatGraf* window is displayed (see Figure 1). Most of the *SkatGraf* commands are issued directly from this window.

Using *SkatGraf*

The software provided with the Skatron machine permits data to be stored in one of two formats: listmode and non-listmode. In listmode files data are stored separately for each cell that is measured, whereas in non-listmode files data are converted into 'counts per channel number' format for each parameter recorded prior to storage. If non-listmode data acquisition is selected (the default) then data are stored in ASCII format (.DAT files) whereas listmode files are stored in binary format (.LST files). *SkatGraf* has been written to process the non-listmode format files only, since binary files may easily be converted into the ASCII format by reading them back into the Skatron data acquisition software and resaving the data in the appropriate format.

Saving the data in listmode format has the advantage that the data can be reprocessed after acquisition. For example gates can be set to include only a specific subpopulation prior to reading in the listmode file. The resulting ASCII format file that is saved will then only contain the data in the region of interest. Alternatively, the same effect can be achieved by selecting the range of data to be plotted in the *gnuplot* package.

The non-listmode files are saved by the Skatron data acquisition software in a format that approximates to the Flow Cytometry File Standard outlined by Murphy & Chused (1984). The first part of the non-listmode data file is a text area (see Figure 2) containing information about the contents of the file, the Skatron settings used for data acquisition (dual-parameter histograms stored, gain and PMT voltage for each parameter, sheath fluid speed, sample speed etc.). Following the text comes the data area where the number of counts binned into each channel for each single- and dual-parameter histogram are stored.

SkatGraf menus are activated either by clicking with the mouse on the appropriate menu titles on the menu bar (Figure 1) or by pressing the **Alt** key followed by the highlighted letter in the menu name. Options can then be selected from the drop-down menus in the same way. Data files are loaded by using the **Select** option from the **File** menu and choosing the file to be analysed from the list box. At this point in the program only the text area of the data file is read to find out what sorts of data are stored in the file and also to determine the identity of the sample.

Graphs of the data from the selected file can then be displayed on the screen by selecting the **Display** option from the **Graph** menu. A dialogue box is shown prompting the user for the type of data to plot (see Figure 3). When the correct data type has been selected and the **Load** button has been pressed a second dialogue box is displayed prompting for axes labelling, graph title and, for three-dimensional graphs, the elevation and rotation angles from which to view the graph.

After a graph has been displayed on the screen it can be printed by selecting the **Print** command from the **Graph** menu. An example of a single and a dual-parameter histogram produced by *SkatGraf* in this way are shown in Figures 4*a* and 4*b*.
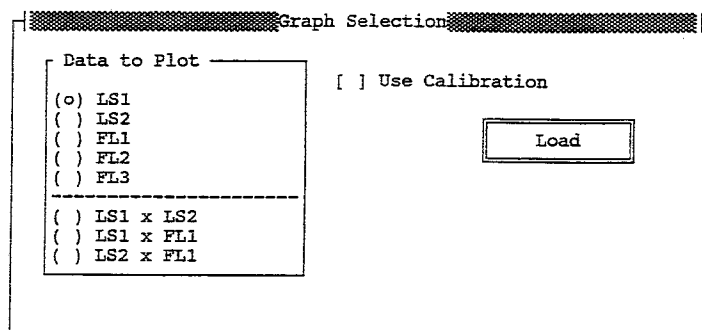
Figure 3 The graph selection dialogue box. All of the histograms stored by the Skatron data acquisition software are listed on the left hand side of the dialogue box.
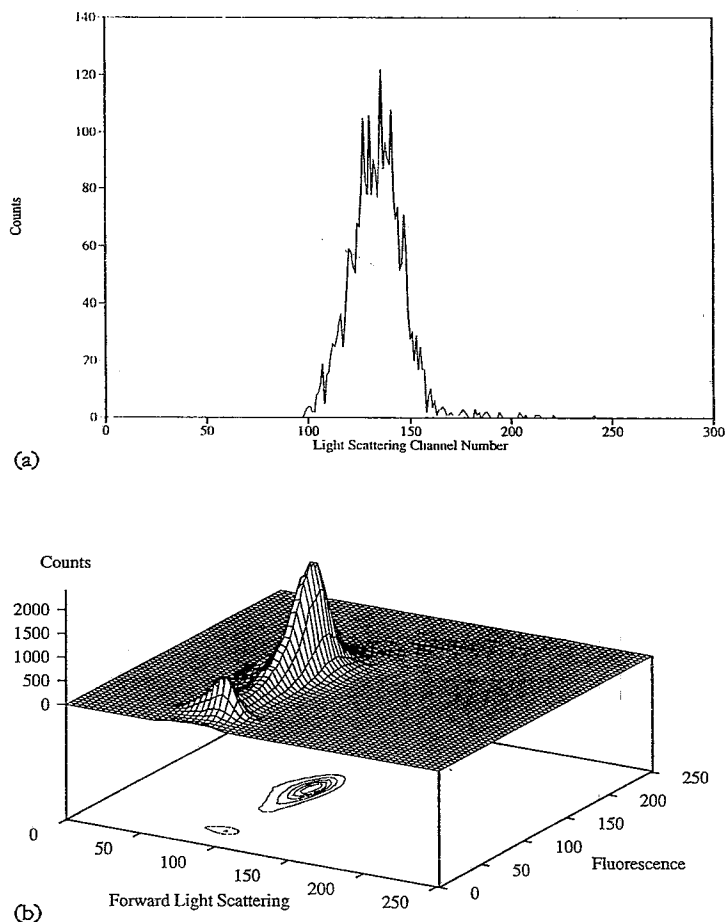
Figure 4 (a) A single parameter histogram showing the distribution of fluorescence of a population of *Micrococcus luteus* taken from a carbon-limited continuous culture growing at a dilution rate of 0.01h⁻¹ and stained with rhodamine 123 [for details see (Davey *et al*, 1993*a*)]. The FITC filter block (excitation 470—495nm, band-stop 510nm, emission 520-550nm) was used for this measurement, and the PMT voltage for the FL1 channel was set to 750. *(b)* Dual-parameter histogram showing the same sample as in A together with the forward light scattering data. The PMT voltage for the LS1 channel was 600.

Using the Calibration Option

It was mentioned in the introduction that it is often desirable to express one's data as absolute values rather than in terms of channel number. Provided suitable calibration samples are run through the flow cytometer *using the same settings as for the cell samples of interest*
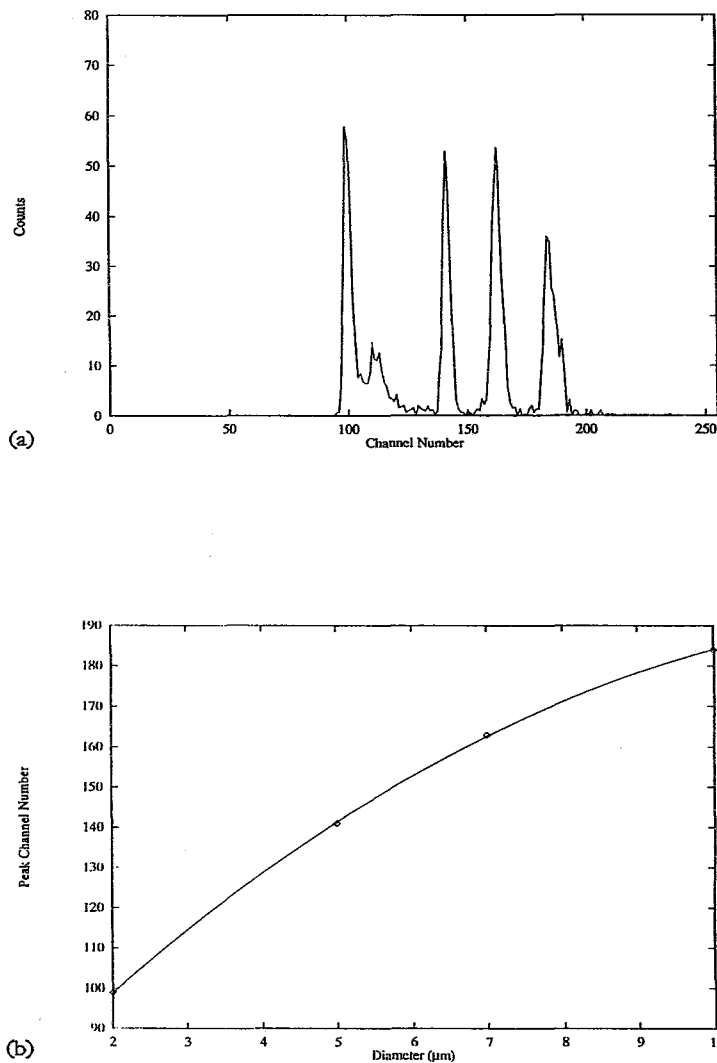
(a)

(b)

Figure 5 (see this page and facing page) (a.) Flow cytometric analysis of the forward light scattering properties of a cocktail of latex beads. The FITC filter block (excitation 470-495 nm, band-stop 510 nm, emission 520—550 nm) was used, and the PMT setting for the LS1 channel was 500. From left to right the major peaks represent beads of 2, 5, 7 and 10 μm diameter respectively. *(b)* Relationship between particle diameter and peak channel number of forward light scatter for latex beads. The data are well fitted by the 3-term polynomial (y = 63.62 + 19.05x + 0.7x^2) where x is the diameter in μm and y is the equivalent channel number.

then *SkatGraf* may be used to convert the channel numbers into absolute values. Figure 5a shows the flow cytometric data from a cocktail of latex beads of known diameter which can be used to determine the diameter of cells of unknown size from their forward light-scattering profile.

Selecting the **Calibration** option from the **Graph** menu causes a table to be displayed. The calibration data are entered into this table and when the OK button is pressed a routine is called that attempts to fit the data to a 3-term polynomial :

$$y = ax^2 + bx + c \qquad (1)$$

where a, b and c are constants, y is the channel number

and x is the calibrated equivalent of the channel number.

The fit is carried out using the Levenberg-Marquardt method of non-linear least squares curve fitting (Bevington, 1969). If the routine succeeds in fitting the equation to the data the best fit values obtained by the fit will be displayed, followed by a graph of the calibration data together with the fitted curve to enable the user to assess the goodness of fit (Figure 5b). When a fit to the 3-term polynomial has been achieved the **Use Calibration** option will become available in the **Graph Selection** and **Save Data** dialogues. When the Use Calibration option is selected the channel numbers are converted to their equivalent calibrated values via the following equation which is the positive solution for the quadratic equation formed by subtracting y from both sides of equation (1).

$$x = \frac{-b + \sqrt{b^2 - 4a(c - y)}}{2aF} \qquad (2)$$

where a, b, c, x and y are as described for the 3-term polynomial [see equation (1)], and F is the additional calibration factor.

Figure 5c shows the forward light scattering properties of a batch culture of yeast in terms of channel numbers. Figure 5d shows the same data set but with the channel numbers converted to diameters using the bead calibration shown in Figure 5a and 5b together with an additional calibration factor which describes the difference in light-scattering properties of latex beads and cells of the same size (Davey *et al.*, 1993b).

Exporting Data for Use in Other Applications

One of the main problems that we have found with the use of files saved by the Skatron data acquisition software is that their format does not allow them easily to be imported into commercially available plotting packages. For this reason we have added a **Save Data** option to the **File** menu of the main *SkatGraf* window (Figure 1). This option permits the data areas of the file to be exported in several formats as outlined below.

- *Uncalibrated single-parameter histograms*
  Two columns of data are exported, the first column contains the channel numbers (0—255) and the second column contains the counts associated with that channel number.

- *Calibrated single-parameter histograms*
  Again two columns of data are exported, the first column contains the calibrated equivalents of each channel number (e.g. diameters) and the second column is as described for the uncalibrated data sets (see above). One disadvantage of using a polynomial is that outside the range of the calibration data use of the fit can rapidly become unreliable. Where this occurs a value of -1000 is placed in the file in place of the calibrated equivalent of the channel number to indicate that extrapolation to this channel number is unsafe.

- *X Y Z format dual-parameter histograms*
  Three TAB separated columns are exported, where

the first column contains the channel number for the first parameter (x), the second column contains the channel number for the second parameter (y) and the third column contains the counts associated with that x-y pair. It is important to note that although 256 channel numbers (0—255) are used for single-parameter histograms, the Skatron data acquisition software averages data over 4 channels for the dual parameter histograms, thus giving only 64 channels for each parameter.

- *Grid format dual-parameter histograms*
  Data are exported as a 64 by 64 matrix with each value in the matrix corresponding to the number of counts at a particular pair of channel numbers. The data are arranged so that the channel numbers for the first parameter increase from left to right across the grid, while the channel numbers for the second parameter increase from the top of the grid to the bottom.

Discussion

Traditional methods of microbiological analysis have either involved measurements of the gross characteristics of populations or the measurement of (small numbers of) individual cells by light microscopy. The development of flow cytometry has led to the ability to measure the fluorescence and light scattering properties of *individual* cells in large numbers at high speeds. As a consequence of the development of such rapid methods the requirement for efficient and easy to use data analysis systems has increased.

SkatGraf has been developed to meet these needs out of necessity, due to the lack of suitable commercial software and because of the problems (caused by the file format) of importing the raw data files into general-purpose plotting packages. It was designed to supersede *Flowtovp.wks* (Davey *et al.*, 1990), a spreadsheet template that permitted calibration of the data. *SkatGraf* has many features that were not present in *Flowtovp*, the most important of which is that *SkatGraf* can take data from its raw format in the original file saved by the Skatron data acquisition software right through to publication-quality hardcopy output within a single program; to create a graph such as that shown in Figure 5*d* would have involved the use of four separate packages if *Flowtovp* had been used. In addition the ability to process dual-parameter histograms has been added to *SkatGraf* over and above the single-parameter histogram processing features of *Flowtovp*.

We conclude that *SkatGraf* provides a convenient and useful means for processing data files acquired with the software provided with the Skatron flow cytometer.
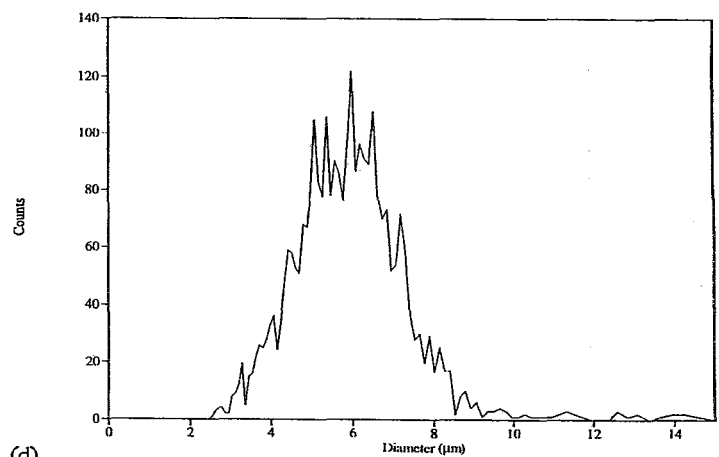
The *SkatGraf* software (1.2 Mbytes) is available in executable form on receipt of an appropriately formatted floppy disk.

Acknowledgements
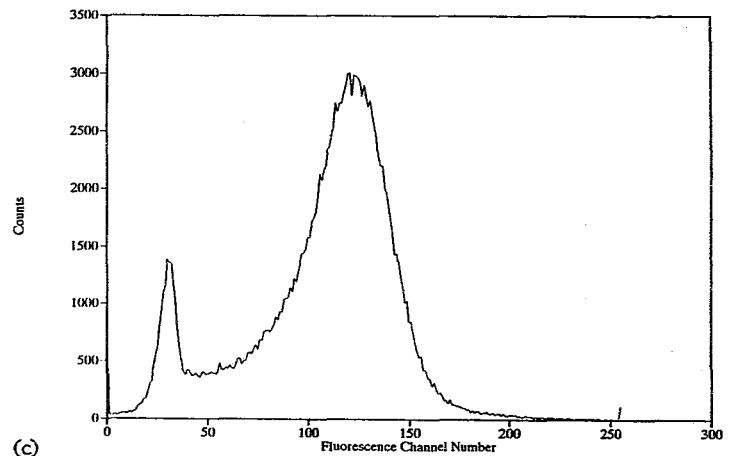
(c)

(d)

Figure 5 (see this page and facing page) (c) Forward light-scattering properties of a population of yeast taken from a batch culture. *(d)* The same data as in *(c)* but the channel numbers have been converted to diameters by *SkatGraf*, using the polynomial in *(b)* along with an additional calibration factor of 0.7627 (see text for more details).

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