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GRAPHICAL REPRESENTATION OF THREE-PARAMETER FLOW CYTOMETER HISTOGRAMS BY A NEWLY DEVELOPED FORTRAN IV COMPUTER PROGRAM

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A Fortran IV computer program is described which displays cell clusters of three-parameter flow cytometer histograms as clouds. A cloud consists of a series of contours at a selected amplitude level. Invisible parts of contours are suppressed to increase the perspective impression of the graph. The perspective plots give a quick and comprehensive picture of the spatial arrangement of cell clusters in such histograms. The program can also display four-parameter histograms, provided that one of the parameters is cell volume. The four parameters are reduced to three parameters by calculating either the cytoplasmic concentration of the labelled substance in single cells or their packing density on the cell surface, or else their photometric absorption per unit cross sectional area. The reduced histogram is plotted as a three-parameter histogram.

Key words: Flow cytometry; three-parameter data representation.

The development of flow cytometers permits measurement of three or four parameters of single cells simultaneously at high speed. An important requirement for the understanding of the results of such measurements is a comprehensible graphical display of the experimental histograms. The three coordinate directions of an orthogonal X, Y, Z coordinate system are fully occupied by three parameters (*e.g.* cell volume, fluorescence 1 and 2) and none is left for the display of channel contents. One way of displaying a three-parameter histogram is to reduce it to three two-parameter histograms either electronically (4) or by software controlled printing (Fig. 1) or plotting (Fig. 2) of cumulated projections of the three-parameter histogram onto the X-Y, X-Z and Y-Z planes. It is, however, difficult to imagine the spatial arrangement of the cell clusters. The display of the three-parameter histogram in a single graph is, therefore, preferable. A cell cluster in a three-parameter histogram can be imagined as a cloud of numbers (Fig. 3A), each number representing the content of an individual channel of the histogram. The clouds can be displayed by indicating channel contents above a given amplitude as light points on an oscilloscope (5). The perspective impression of such graphs is, however, not optimal. Another method of displaying cell clusters of three-dimensional histograms is to plot contours for a given amplitude (Fig. 3B) because contour plots give a better perspective resolution of the cell clusters. The computer program presented in this paper realizes contour plots of three dimensional histograms. Contour plotting raises, however, the problem of suppressing invisible parts of the contour. Computer programs for the suppression of invisible lines exist as algorithms nr. 420 and 483 of the collected algorithms of CACM (7, 8). These algorithms can not be used for contour plotting because only one Z-coordinate is allowed for each X-coordinate. Contours have, however, mostly two or more Z-coordinates per X-coordinate. Algorithm 475 (9) overcomes this difficulty. It generates

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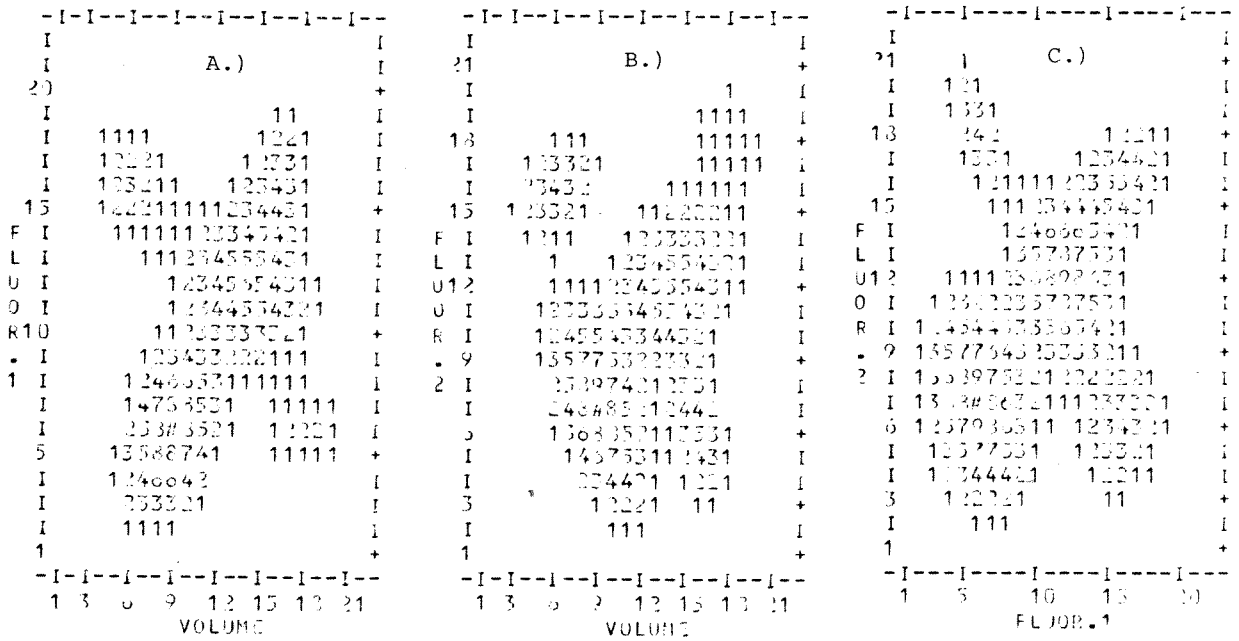

Fig. 1. Isoamplitude print of the cumulated projections of a three-parameter histogram on the X-Y (A), X-Z (B) and Y-Z (C) plane. The numbers 1 to 9 represent the channel contents in steps of 10% after normalization of all channel contents to the highest amplitude (#).

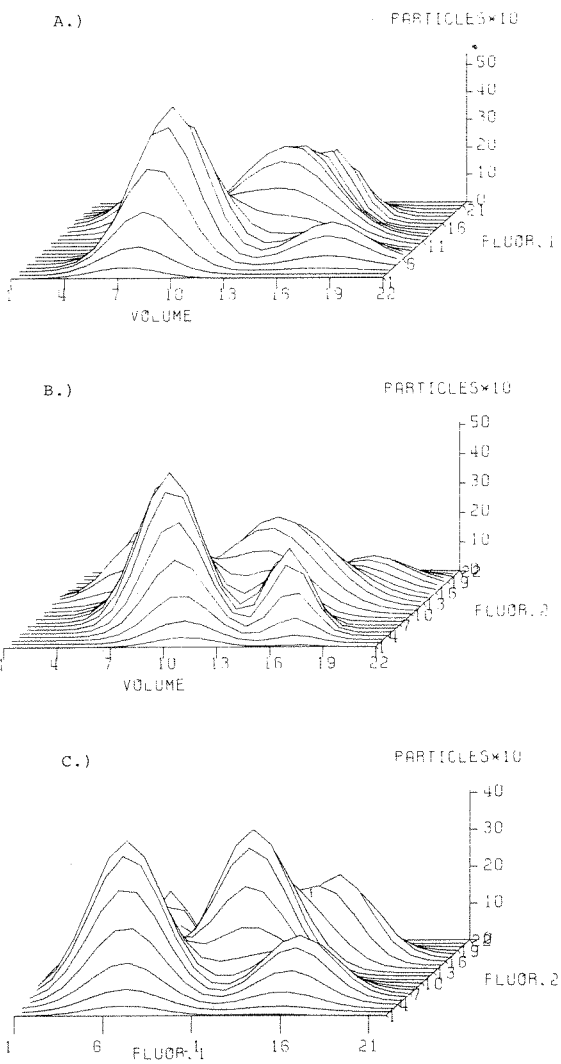

Fig. 2. Perspective plot of the cumulated projections of Fig. 1.

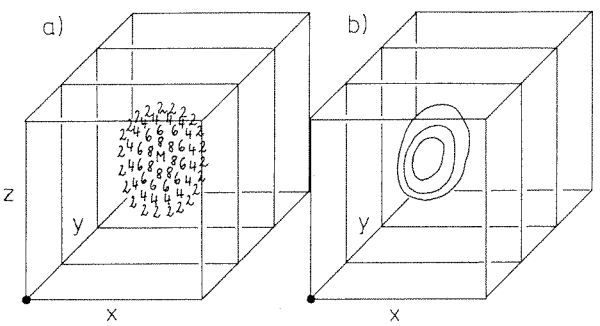

Fig. 3. A cell cluster in a three-parameter histogram can be represented as a cloud of numbers (A) which stand for the channel contents of the histogram. Drawing lines between channels of equal content gives a contour plot (B). The cell cluster is visualized as a cloud.

a reference image of the contour area within a lattice array of the computer memory. Lattice points within the contour area are set to 1 while those outside are set to zero. The program checks the reference image each time a new piece of the contour is to be plotted. The contour piece is plotted if the program finds zero in the reference image, it is suppressed if one is encountered. The difficulty of algorithm 475 is that only contour pieces with integer start and end coordinates can be plotted. This means that straight lines or circles are plotted as stairlike curves. They are not precise replicates of the original shape of the contour. The number of lattice points can be increased to decrease this imprecision.

A large core memory is, however, required to obtain smooth contours. The reference lattice should contain 1000 × 1000 points if the plot has a size of 10 × 10 cm and if 0.1 mm distances are to be resolved.

The aim of the program described here was to avoid these difficulties. The newly developed program is capable first: of maintaining a 0.1 mm resolution in a 10 × 10 cm plot with a comparatively small lattice array of 100 × 100 points, and second: of drawing smooth contours using floating point coordinates instead of the less precise integer coordinates. The results (Figs. 4, 5) show that perspectively well discernible and smoothly limited clusters can be plotted with the program.

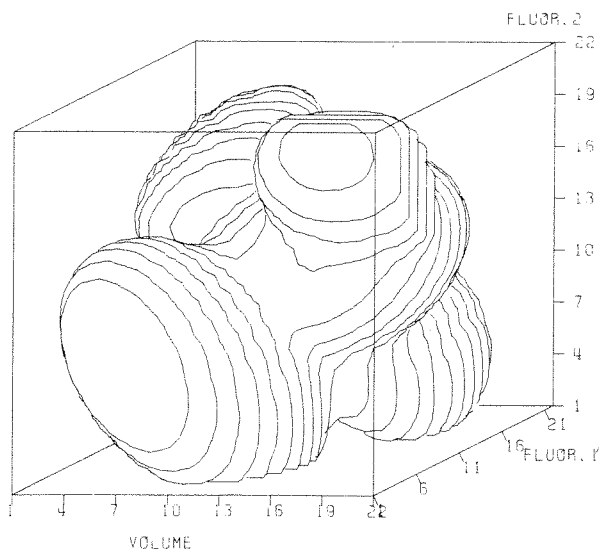

Fig. 4. Overall cloud dimensions of five superimposed three-dimensional gaussian normal distributions (contour level 1% of maximum amplitude).

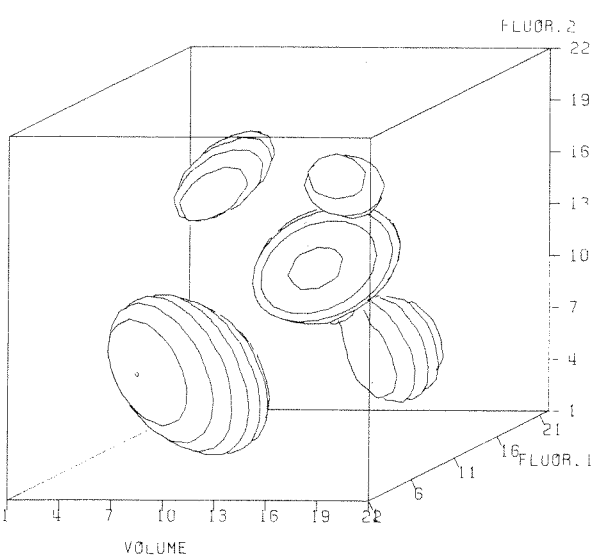

Fig. 5. Spatial resolution of the individual cell clusters of the histogram of Fig. 4 by plotting the 20% contour level of Fig. 4.

PROGRAM CONCEPT

The program uses a two dimensional reference image of the contour areas. The reference image consists of a lattice array of 100 × 100 points in the computer memory. An information of 16 bits can be stored at each lattice point. This allows storage of the number of the contour area and of additional information on the coordinates of the border points in the lattice array.

The lattice array is zeroed and the three-parameter histogram is normalised to its maximum channel content at the beginning of the program. The contour line of the first X–Z slice of the three-parameter histogram is calculated from the normalized channel contents (Fig. 3A) by a contour subroutine (2). The subroutine delivers the floating point X, Z coordinates of a sequence of contour points at a preselected amplitude level. These points, interconnected by straight lines, form the contour and enclose the contour area. Lattice points inside the contour area are set to the number of the X–Z slice which is equal to the Y-coordinate of the three-parameter histogram (Fig. 3B). Setting the lattice point to a non-zero number indicated that this location is forbidden for penetration by later contours. The lattice points which are border points of the contour, contain the first digit behind the decimal point of the X, Z-floating point coordinates in encoded form besides the slice number. An additional number is encoded in the content of the lattice point which indicates if the point is an upper, lower, left or right border point of the contour. The reason for encoding this information is that the original floating point coordinates can be reconstituted later from these items of information. A 0.1 mm resolution between two contours is achieved by this procedure. The program plots the first contour line as soon as all border points of the contour area have been labelled. It proceeds then to the second contour. The program sets all visible lattice points within the second contour (*i.e.* lattice points which are zero) to the slice number of the second area. This indicates that for further contours these locations are forbidden. The border point data of the second contour area are encoded in the same way as for the first contour, provided the border point is visible. A border point is visible if the content of the reference lattice point is either zero or equal to the slice number of the second contour area. The program may encounter border points of the first contour area while encoding the border points of the second area. In this case the program decodes the coordinate and border information of the previous border point from the reference lattice and compares it with the coordinates of the new point. The coordinate information is replaced if the new point is visible. The old coordinate information is conserved if the new point is invisible. When an invisible border point is encountered, all visible points of the contour so far accumulated are plotted by intercon-

necting them with straight lines. The program then searches another sequence of visible contour points. The program proceeds to the third contour area when all visible contour points of the second area have been plotted. The third and following contour areas are processed in the same way as the second contour area until the last X–Z slice is terminated. A cube is plotted around the cell clusters to indicate the full extension of the three-dimensional histogram. The program can turn the three-parameter histogram by 90, 180 and 270 degrees around the Z or X-axis. In addition each contour area can be shifted with respect to the previous contour area by one or multiples of one lattice point to the left or right or upward. The turn and shift operations visualize details of the three-parameter histogram which otherwise might remain invisible. The program can also print two-parameter isoamplitude graphs (Fig. 1) and plot perspective graphs of the cumulated projections of the three-parameter histogram on to the X–Y, X–Z and Y–Z planes (Fig. 2). The perspective two-parameter plot was described earlier (6). It uses the hidden line algorithm 420 (8). The compiled program with all plot routines included requires 260 kbyte core memory and is at present implemented on a Siemens 4004/150 *(Siemens,* Munich, Germany) and on a Vax 11/780 computer *(Digital Equipment Corporation,* Maynard, Mass.). The graphs are drawn by a Calcomp 936 or 1012 plotter *(California Computer Products,* Anaheim, Cal.). The three-parameter histograms are obtained by a two step procedure. The first step is the data acquisition. The analogue values of the three parameters of an individual cell as analyzed by the flow cytometer (3) are digitized with a resolution of 128 steps and stored on-line at a speed of 6 kHz via an Interdata 74 computer (*Interdata Corporation,* Oceanport, New Jersey, 64 kbyte core memory) on magnetic tape (1). The sequential list mode data on the magnetic tape are classified in a second step as a 22 × 22 × 22 matrix by a Fortran IV computer program (1) in the same computer. This three-parameter histogram is ready for graphical display with the contour program. The reasons for the choice of a low resolution 22 × 22 × 22 matrix for the display of the three-parameter histogram was the difficulty of adequately filling a three-parameter histogram with more channels in the X, Y and Z directions (*e.g.* 64 × 64 × 64) at a flow cytometer analysis rate between 1000 to 1500 particles/s. The classifying program has the option of classifying from 1 to 6 resolution steps of the digitized primary data into one class of the 22 × 22 × 22 three-parameter histogram. This compensates to some degree for the low resolution of the 22 × 22 × 22 matrix, because it allows one to cut out interesting parts of the three-parameter histogram and to display them with a better resolution.

PROGRAM USE

Several clusters were generated for test purposes in the three-parameter matrix by superimposing five threedimensional gaussian normal distributions. A first step in the evaluation of the cluster pattern was to determine the outer limits of the clouds by plotting the 1% contour level (Fig. 4). The individual cell clusters were, however, better segmented and more easily distinguishable at a contour level of 20% of the maximum amplitude (Fig. 5). One can readily determine from the 20% contour plot whether clusters are present or absent. The quantitative estimation of the location of the centre of a cell cluster is difficult from Figs. 4 and 5. A better way is shown in Fig. 6, where the three dimensional

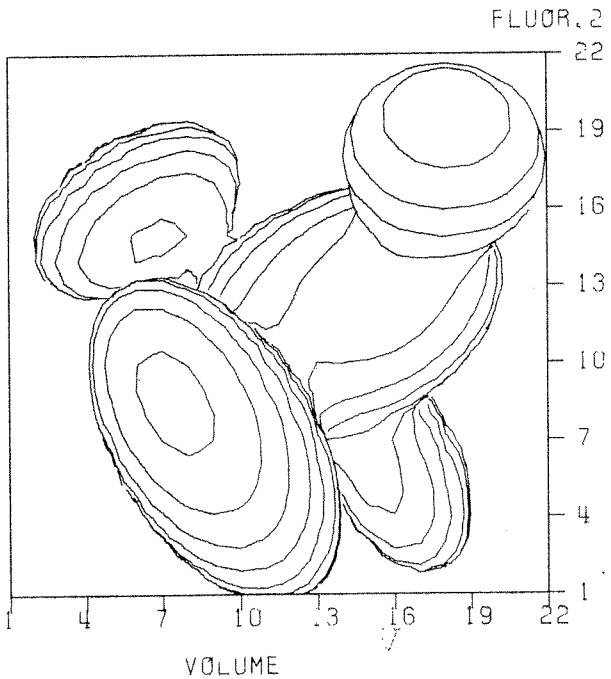

Fig. 6. Reduction of a three-dimensional contour plot to a two-dimensional plot (contour level 5%).

plot is reduced to a two-parameter plot. The coordinates of the centre of the cluster and the angles of inclination of the cluster towards the coordinate axes can be accurately determined without calculations. This is important when the starting parameters for fits of three-parameter gaussian distributions to the experimental histogram have to be estimated.

The program can also handle four parameters if one of the parameters is cell volume. The four parameters are reduced to three biochemically defined parameters by calculating average concentrations for cytoplasmic substances, surface packing densities for cell

membrane associated parameters, or light absorption per unit cross sectional area for histochemical reactions. The calculated parameters are of interest for quantitative biochemical studies on the single cell level. Experimental results on different cell populations or cells in various metabolic states become comparable in a standardised way if the four to three-parameter reduction is performed.

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