

MFI: a flow cytometry list mode data analysis program optimized for batch processing under MS-DOS¹

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¹This work was supported in part by grant DCB-9017743 from the National Science Foundation.

This manuscript, written in 1993, was never published. The primary citation for MFI is:

**Martz, Eric. 1992-2001. MFI: a flow cytometry list mode data analysis program
optimized for batch processing under MS-DOS.
<http://www.umass.edu/microbio/mfi>**

The manuscript has been revised to match the 1996 ("final") version of MFI.

ABSTRACT

Some software provided with flow cytometers offers high flexibility but is inefficient for obtaining corrected fluorescence intensities from large numbers of files. A program optimized for batch-mode calculation of corrected **Median Fluorescence Intensities**, named **MFI**, was written in the C language for operation under MS-DOS on personal computers. MFI can process 100 list mode files in a few minutes. Results are printed in compact tabular form, and include median fluorescence intensities, gated on 1-3 parameters, converted to a linear scale where logarithmic amplification was employed, corrected for control(s), and converted to molecules of equivalent soluble fluorochrome if desired. Dot plots and histograms can be displayed/printed when necessary; however, tabular output includes values designed to warn should the event-cloud drift relative to the scatter gate. Also, when multiple peaks occur in a histogram, they are automatically detected and values are printed for each peak. These features make it unnecessary to scrutinize graphics for every sample; rather, after the results have been tabulated, one can tag unusual files for graphic display and interpretation. MFI can also convert FCS files to ASCII list mode or histogram data files, and slice a single list mode file for kinetic analysis. The program is freely downloadable; source code is available on request.

KEY TERMS: MS-DOS, personal computers, median fluorescence intensities, kinetics by time slicing, data conversion to ASCII

INTRODUCTION

Many kinds of flow cytometric analyses, such as antibody titrations, require calculation of gated and corrected fluorescence intensities for large numbers of samples. The "Research Software" provided by Becton-Dickinson with their FACScan and FACStar Plus instruments until 1989 has high flexibility, but cannot be configured to print corrected fluorescence intensity results in an automated batch manner; continual operator intervention is required. Processing of hundreds of samples thus consumes an inordinate amount of time. There is no way to print compactly the final values; this encourages manual recording and the possibility of recording errors. Logarithmically acquired parameters are not converted to a linear scale, and there is no provision for subtraction of control values or conversion to molecules of equivalent soluble fluorochrome. Such corrections must be performed manually as a separate task. These limitations prompted me to design a list mode data file analysis program optimized for batch processing of groups of files, with the goal of making the program and its source code available for others to use or enhance as needed.

Principles guiding design of the program were as follows. Once configured, the program should be able **to process a large number of files automatically, without operator intervention**, printing the results in a compact tabular form. The output table should be able to be sent to the screen, the printer, or to a disk file. The table should include any arbitrary subset of the parameters present in the list mode data file, in any arbitrary order. **The output should warn of problems such as drifting of the event cloud out of the scatter gate, or unexpected subpopulations in a cell population thought to be uniform. The output should include a summary of high and low values at the end of a run**, so that, for example, the range of the percentage of events within the gate is easy to determine. **Such warnings would make it unnecessary to examine histograms and dot plots for every file** -- rather, graphics would need be examined and interpreted only when unexpected problems arise. Commonly needed operation modes should be achieved with minimal numbers of keystrokes. The default configuration should be customizable. The program should automatically remember the user-selected configuration settings so that each session begins with the configuration in effect for the previous session. It should be possible to tag an arbitrary subset of the files on the disk and process them in any arbitrary order; this allows any file to be designated as a control, so that its median values could be subtracted from those of other files. The program should be able to remember numerous runs (tagged subsets of the files in a given directory), and each run should display a description. **A description or label should be able to be inserted into each data file**, and the entire contents of the FCS "text" section (keywords plus associated values) should be readily displayed for any file.

Gate boundaries should be able to be set on any one, two, or three parameters, thus allowing gating on two scatter parameters plus, for example, propidium iodide

simultaneously. A number of gates should be able to be remembered at once, along with descriptive names. Any gate should be able to be applied to any file or series of files.

While graphics were not the major goal of this program, the following graphics capabilities were considered essential. The basic graphics screen should be able to show separate histograms for up to six parameters, plus a dot plot, all on a single screen. Each histogram should be capable of overlaying results from up to two previous arbitrarily selected files. It should be easy to overlay histograms from different gatings of the same file. Overlaid histograms should be scaled to a common scale by default, so that their areas are proportional to numbers of in-gate events; alternatively, each overlaid histogram should be able to be scaled independently. Pileups of off-scale events in end channels should be shown by default, but optionally clipped to enable raising the middle of the histogram to full-scale. It should be possible to smooth histograms to any degree desired. Any two parameters should be capable of becoming the axes for a dot plot. Gate boundaries should be able to be set interactively on a graphics display of the dot plot. Dot plots should display any arbitrarily selected subset of the gates defined, indicating which gate is currently in effect. It should be easy to magnify any single histogram or dot plot to fill the entire screen. Each graphic should be unequivocally labeled to show the name of the file containing the data, and the gate used (if any). Any graphics screen should be printable on any common printer.

Gated list mode or histogram data should be exportable to ASCII files suitable for import into other software, such as spreadsheets or publication-quality plotting programs. When time-slicing is requested, each slice should be exportable as an ASCII file.

For kinetic analysis, the program should be able to slice a single list mode file into an arbitrary number of segments of consecutive events, reporting intensities and graphics on each slice.

An important overall design principle was that the program should minimize the possibility of inadvertently misleading the user. This requires particular care in the areas of warning when event clouds drift out of a gate, warning when instrument settings were changed between files, management of end-pileups of off-scale events, and vertical scaling of overlaid histograms.

Finally, the program should have extensive built-in help, and a built-in tutorial. Although it should not *require* a color monitor, a mouse, nor Microsoft Windows, it should use colors when available, and run in a DOS window under Windows.

METHODS

Cytometers supported. The flow cytometry standard for list mode data files (1) facilitates support of many cytometers and data acquisition software packages. Hundreds of FACScan and FACStar Plus files acquired with the corresponding "Research Software" (released in 1987-1988 by Becton-Dickinson, Mountain View CA USA; Hewlett-Packard Pascal 3.1 operating system) have been tested. At least one data file from each of the other combinations listed below appears to be compatible with MFI:

Becton-Dickinson FACStation 1.0 "CellQuest" data acquired under Macintosh OS.

FACScan, FACStar Plus, and FACSVantage data acquired with Lysys II (versions 1.0 11/90, 1.1 2/92, or 1.2 8/92 from Becton-Dickinson; Hewlett-Packard Pascal 3.2 operating system), or Consort 30 (version F, 2/88).

Becton-Dickinson FACScan data acquired with CellFit (CRDNA) Software 1.0 under Hewlett-Packard Pascal 3.1 operating system.

Becton-Dickinson FACS 440 with B-D software operating under DOS 3 or VAX/VMS version 4.7.

Coulter Elite cytometer with Coulter software operating under DOS 4 or DOS 5; Coulter Epics XL under DOS 5, Coulter Epics MDADS/86 operating under DOS (Coulter Corp., Hialeah FL USA).

Sample data file provided with WINLIST 1.0 from Verity Software House (Topsham ME USA).

Cytomation Inc.'s CyCLOPS software.

Data acquired on the Coulter Epics Profile under DOS is not stored in "Flow Cytometry Standard" file format and cannot be processed by MFI. A conversion program is available from Verity.

Source Code. Source code was written in ANSI C, and exceeds 25,000 lines in length. Some debugging employed Instant C (Rational Systems Inc., Natick MA USA). Production compilation and linkage employed Microsoft C 5.1 (Microsoft Corp., Redmond WA USA) and later Borland C++ 3.1. The final executable files were compressed with the program LZEXE.EXE (ver. 0.91) generously placed in the public domain by its author, Fabrice Bellard (Grabels, France). This reduces the sizes of the MFI executable files by more than two thirds; decompression occurs transparently in memory at execution time. Because the program became too large to fit into the DOS 640K memory space, it was split into a parent program MFI.EXE, and child programs (e.g. MFIHELP.EXE). When a child program is called, the parent's memory image is moved to extended or expanded memory (or disk); when the child program exits, the

parent is restored and its execution resumed. Child calling is managed by Hold Everything (tm) (South Mountain Software, South Orange NJ USA).

Libraries. High level code was isolated from the compiler's library with a "uniform input/output" interface library. Porting the program to a different MS-DOS compiler should thus require changes only in the uniform input/output library. DOS specific functions were isolated to a DOS interface library. Graphics and Savitzky-Golay smoothing were implemented with the library "Science and engineering tools for Microsoft C" (IPC-MC-006, Quinn-Curtis, Needham MA USA). Modification of source code by others will require purchase of licenses for the Quinn-Curtis and Hold Everything libraries.

Availability. The executable MFI program is freely downloadable at <http://www.umass.edu/microbio/mfi>

Source code (over 20,000 lines) is available on request, provided the recipient agrees to make derivative programs available via FTP to the scientific community at large, free of charge.

Transfer of data files from Hewlett-Packard to DOS. [In 2001, this section is largely of historical interest.] At least four methods are available for transferring list mode data files from Hewlett-Packard computers, which acquire the data from many flow cytometers, to MS-DOS computers. (i) Data written to three and one-half inch diskettes can be read on DOS computers using commercial software. This method is too slow and cumbersome for routine use. (ii) Data can be transferred using serial ports and public domain software. This is also very slow; several dozen data files take hours to transfer. (iii) Data can be transferred from the IEEE-488 GPIB of the Hewlett Packard (the thick cable that connects each subunit of the H-P computer) to an IEEE-488 card installed in the PC bus. HPPCLINK programs for managing the transfer were written at Becton-Dickinson and contributed to the public domain. This method requires about 20 sec to transfer a 50K file. Our card model GPIB-PC2 came from National Instruments (12109 Technology Blvd., Austin TX 78727). Further information generously provided by David Coder is available in a file HPPCLINK.PS in /pub from the ftp site mentioned above. (iv) If the Hewlett-Packard computer is equipped with a network interface, data files can be transferred by FTP. This is the fastest method. Once the data files are on an MS-DOS computer, they can be moved to other MS-DOS computers efficiently by network FTP.

RESULTS

All of the design objectives specified in the introduction have been achieved in MFI. In batch mode with output to a disk file, processing 100 files of 5,000 events each takes about 7 minutes on an 80386 cpu with a math coprocessor running at 25 MHz (4

sec/file), or about 2.3 minutes on an 80486DX running at 33 MHz (1.4 sec/file).

Input file selection, gate assignment, and run organization. MFI begins by displaying a sorted list of all the data files in the current disk directory (non FCS files are omitted) as shown in Figure 1.

```

4DOS - MFI
DATA FILE RUN ORGANIZATION MFI: Median Fluorescence Intensity ver. 3.4kB8
Run 2: Titration of TS1/18 Ascites on JY1 Cells
0 GDD49001  ▶" GDD49013
1 GDD49002  ▶" GDD49014
"CH GDD49004  ▶" GDD49015
" GDD49006  2[ GDD49003
" GDD49007  "CH GDD49005
" GDD49008  " GDD49028
" GDD49009  " GDD49029
" GDD49010  " GDD49030
"H GDD49011  " GDD49031
" GDD49012  " GDD49032

#LABEL: FITC Standard Beads #3, 212 KMESF

↑ ↓/Space → ← Home End 8 of 20 files are tagged.
ENTER to accept & proceed.
▶Tag/Untag filename Runs/+/ - Save cfg. Control *
Begin/End here Label/label keyword/@ Histogram overlay (2 max)*
All: tag/untag/clear/* Move block of filenames [ discontinue prior C, H *
0-9, " gate/ditto & copy filename Omit filename
F1 Help/Gates/Keywords All labels*/Prompts* (*toggle)
Quit to DOS Freshen list from disk
  
```

Figure 1. Input file run organization screen of the MFI program. This example shows a series of files from an experiment designated GDD49, including files 001-032 (additional files continue on the next "page" accessed by pressing the PgDn key). One file is always highlighted (green bar over file 001), and the #LABEL line pertains to this file. The highlight can be moved with the keyboard arrow keys. The bottom of the screen shows in boldface command keys that activate various operations. Should files be removed from or added to the current directory, the Freshen command updates the file list, maintaining all file marks. See text for further explanation. A more detailed explanation is available in the on-line tutorial (www.umass.edu/microbio/mfi).

As few as one, or as many as several hundred files can be managed by MFI in a single directory. Subsets of files can be tagged and rearranged for processing in any arbitrary order. Note that files 3 (GDD49003) and 5 In Fig. 1 have been moved from their original alphanumeric order into a position prior to file 28. (They are control files for a different cell type that first apply to file 28).

MFI remembers up to 16 separate runs for a given set of files. A "run" is a tagged subset of the files to be processed to generate tabular output. For the

experiment shown in Fig. 1, Run 1 (not shown) included only file 1, a sample of standard beads. Run 2, shown in Fig. 1, has 8 files tagged for processing (marked ➤). After these 8 files have been processed, one can return to this run organization screen and tag a different subset of files for the next run. One can move up or down to adjacent run numbers with the '+' and '-' keys, or press 'R' for a selection menu showing all run descriptions.

The number **0** next to file 1 instructs that it is to be processed ungated; the number **1** next to file 2 indicates that gate 1 will be applied to it. (The gate limits need not be defined at the time the gate number is assigned; in this case, a graphic gate-setting screen appears automatically when the file is processed.) The **ditto** marks for subsequent files instruct that the most recently specified gate be applied to them; similarly, gate **2** will be applied beginning at file 3, which represents a different cell type. File 4 has been marked with '**C**' as a control file; this means that its fluorescence intensity will automatically be subtracted from those of the subsequent files (until a '[' mark is encountered). The '**H**' instructs that the file's histograms will be remembered and overlaid onto those of subsequent files.

If one file needs to be processed more than once (e.g. for overlaying histograms from different gates applied to the same file) or if it is convenient to have a filename appear at more than one place in the run organization list, its name can be duplicated in the list by pressing the '&' key, then moved as desired.

Data file labeling and FCS keywords. A one-line descriptive label can be created for each list mode data file. This is written into the text block of the data file in an FCS-legal manner, using either a public FCS keyword (e.g. \$COM) or a private keyword (#LABEL is the default). This is the only modification that MFI makes to list mode data files. (Such modified data files remain able to be processed by FACScan Research Software, 2/88, HP DOS 3.1). All other MFI configuration information is stored in the file MFI.CFG (a plain text file).

The label keyword is user configurable. This allows the line in Fig. 1 marked "#LABEL:" to display the value associated with any FCS data file keyword; modifications are prevented for keyword values that affect list mode data integrity (e.g. \$CYT [cytometer that acquired the data], \$DATE, \$GATING, \$PAR [number of parameters saved], \$P1B [bits per data value], \$TOT [events saved], \$TR [threshold]), but permitted for those that do not (e.g. #LABEL, \$COM, \$INST, \$OP, \$PROJ, \$SAMPLE, \$TESTNAME). The entire contents of the text block of the current list mode data file can be displayed by MFI to assist in selecting a label keyword.

Text output. Figure 2 (see separate document [fig2.pdf](#)) shows the text output resulting from the run organization shown in Fig. 1. (The colors in Figure 2 were added to make the organization clearer -- these colors are not in the MFI output.)

Normally, standard beads and cells would be processed in two separate runs; but for illustration purposes, file 1 (beads) has been included in the run shown in Fig. 2. In Fig. 2, lines 23-24 are the column headings for the numeric results that appear (following lines 31-32 identifying the data file) in line 33. Thus the numbers in lines 33, 54, 63, etc. line up with the column headings in lines 23-24.

File 1 (lines 31-32) represents a sample of standard beads with a fluorescence intensity calibration of 212 kilo-molecules of equivalent soluble fluorescein (KMESF) per bead. MFI has determined the median FL1 channel to be 543 (line 33), and converted this to a linear equivalent of 132 ($10^{(\text{median channel}/256)}$, for 256 channels/decade) in line 34. (Linear values are not calculated for FSC and SSC since these parameters were not acquired with logarithmic amplification.) When gated, the value was 135 (not shown). A manual calculation was then made, $212/135 = 1.57$. The latter value was entered in MFI's parameter configuration menu (not shown) as the conversion factor from linear FL1 intensity to KMESF, and the files were reprocessed with MFI to produce the output shown in Fig. 2 (cf. line 26). Separate KMESF factors can be entered for each fluorescence parameter in multicolor work.

In Fig. 2 lines 36-42, MFI has automatically detected two peaks in each of the ungated SSC and FL1 histograms from file 1 (cf. Fig. 4). 19% of the events had low SSC but a wide range of FSC (air bubbles?). The bimodality is reflected in the mean/median ratio of 0.90 (5th value in line 33) for the entire file (before separating the two peaks). This ratio indicates the direction and approximate degree of skew; it is labeled the "spew" within MFI.

6% of the events (Fig. 2, line 42) were twice as bright in FL1 as the remaining 94% (line 41), evidently aggregated pairs of beads. Note the caution message in line 39 stating that for FL1, 18% of the events were in channel 0, that is, at or below the low end of the intensity scale (cf. Fig. 4). In this case, these were the suspected air bubbles, but they illustrate the kind of caution message MFI emits when a configurable percentage (default 10%) of the events fall off-scale (either high or low). Since MFI cannot determine whether such off-scale end-pileups represent a separate peak, they are not reported as a separate peak. The overall median FL1 channel, 543 (line 33), is relatively insensitive to the minor subpopulations: gating out the bubbles and aggregates (not shown) changes it only 2 channels, while it changes the mean 16 channels. (An MFI option to report the mean was turned off for the run shown.) This is why medians are preferable to means.

In a preliminary run, scatter limits for gate #1 ("JY Cells") were set graphically. The gate change requested on the run organization when the number '1' was placed next to file 2 is reported in lines 44-45. In lines 47-50, MFI warns that instrument parameters were changed between files 1 and 2. (In this case, the detector and

amplifier gains were changed for FSC and SSC. Compare with the initial report in lines 27-29.). 62% of the events in file 2 fell within scatter gate #1 (line 63). Addition of second antibody alone added no significant intensity to autofluorescence (4.79 to 5.01 KMESF, files 2 vs. 4). The fact that file 4 was marked 'C' for control is reported in line 59 and reflected in line 66.

Files 11-15 were acquired on samples incubated with a series of 3-fold dilutions of anti-CD18 antibody starting at 1/300 and ending at 1/24,300. The resulting corrected KMESF's from lines 74, 82, 90, 97, and 104 in Fig. 2 are plotted in Fig. 3, where the saturation intensity and half-saturation concentration are determined. (Fig. 3 was produced with a publication-quality graphics program.)

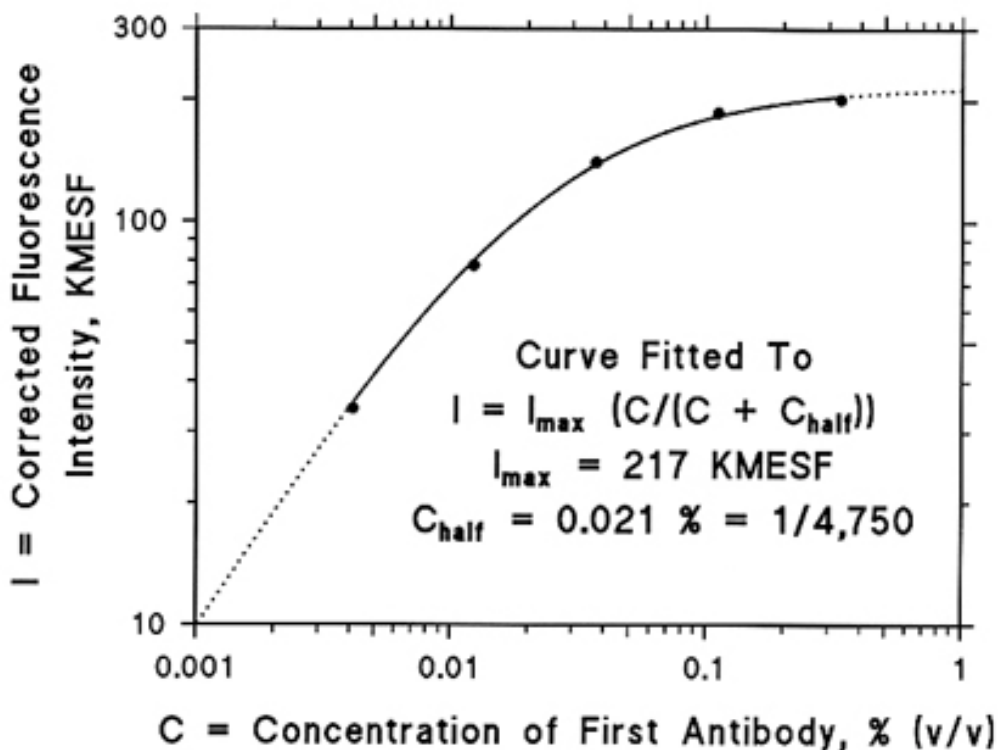


Figure 3. Corrected KMESF values from Figure 2 were fitted to the equation shown (one-site binding Michaelis-Menten equation). This method of determining the concentration giving half-saturation is less sensitive to errors in the lower intensity values than the Lineweaver-Burke method; low intensity points contain larger errors as they diminish to values near the blank (5 KMESF in this case). This figure was created with Fig P (Biosoft, POB 10938, Ferguson MO 63134), which includes the ability to fit data to user-created equations.

MFI's text output ends with a summary of high and low values (lines 109-121, Fig. 2). A quick glance here reveals whether problems with gating occurred. The range of percentages that fell in the scatter gate give an indication of whether the event cloud

drifted in files subsequent to the one on which the gate was set (as can happen, for example, when living cells are warming up to room temperature after being taken off of ice). More sensitive indications of event-cloud drift are given by the R/T and H/T ratios, which are the percentages of the events that fell in the right half and the top half of the gate, respectively. The range of "spew" (mean/median) values (in parentheses in lines 85 and 89) indicates whether serious skewing occurred.

Although only three parameters are shown in Fig. 2 (FSC, SSC, and FL1), up to 8 parameters can be shown simultaneously (two rows of 4 columns each) in MFI's output listing of results. (Notice the note in line 18 regarding parameters 5-8.)

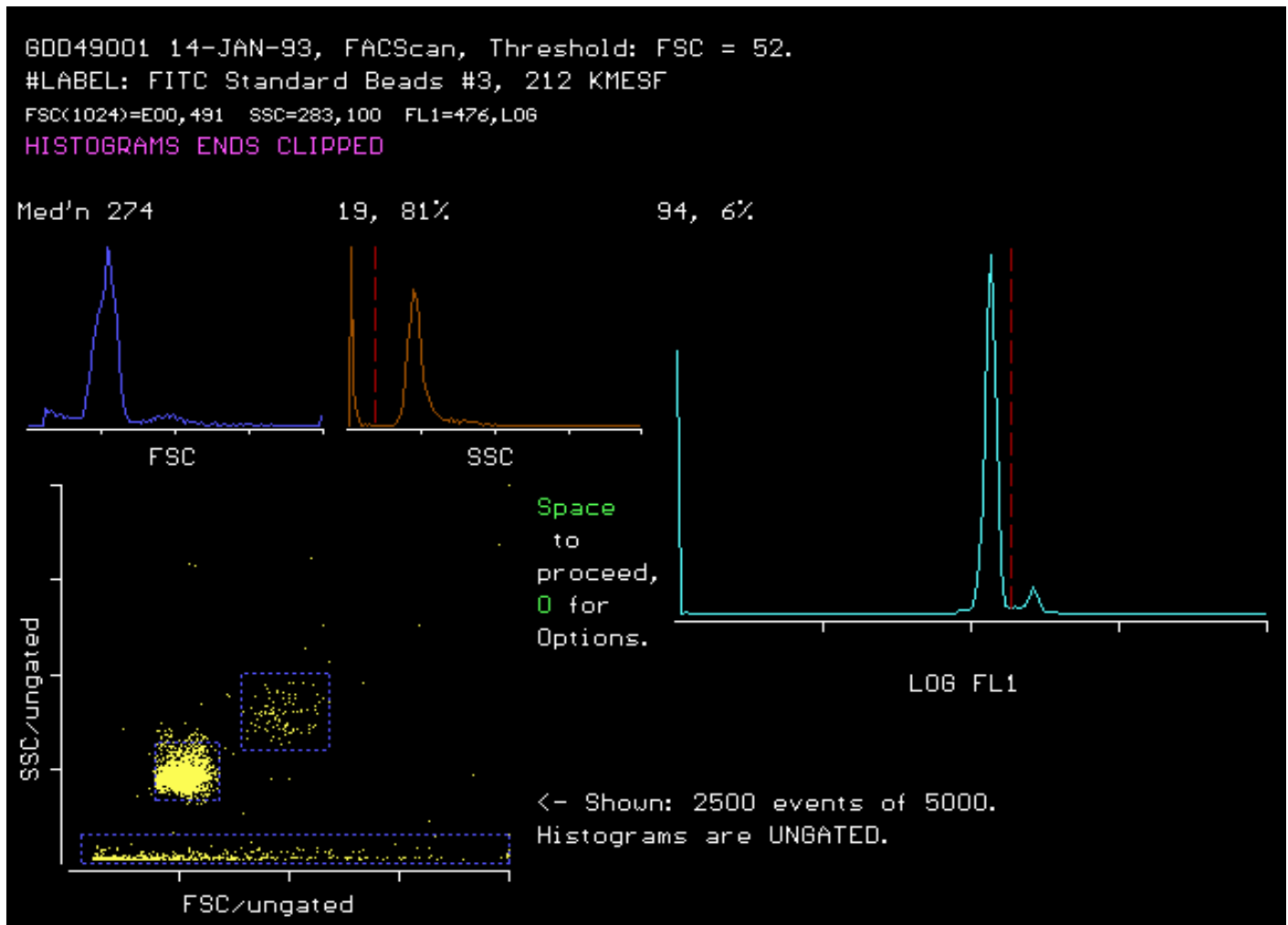


Figure 4. MFI's summary graphics screen for an ungated sample of standard fluorescent beads (#3, Flow Cytometry Standards Corp., POB 12621, Research Triangle Park NC 27709 USA). Three gates are defined but none were active for the histograms shown. The contents of each gate are displayed in Fig. 6.

Graphics. Fig. 4 shows MFI's summary graphics screen; in this example, the file is GDD49001, standard beads. Any one histogram, or the dot plot, can be magnified to fill the entire screen. Three gates are defined and displayed, although the histograms are shown ungated.

In Fig. 4, the red dashed vertical lines in the SSC and FL1 histograms show where MFI found the low points that separate peaks. The sensitivity of peak detection can be adjusted on a configuration menu (not shown). When it is increased sufficiently, three peaks are reported for the FSC histogram in Fig. 4.

Some software, such as FACScan Research Software (2/88), draws vertical lines to represent the Y axes on histograms. This makes it difficult to see a pileup of off-scale events in the first or last channel, which could lead to misinterpretation of data. MFI is designed to make end pileups obvious and minimize the chances of misinterpretation. No Y axis lines are drawn on histograms, so a large number of events in the first or last channel become obvious. By default, histograms are scaled vertically to include end pileups. When an end channel contains a large number of events, this causes the tallest peak in the middle of the histogram to fall short of the top of the graph, making the magnitude of the end pileup obvious. An option is provided to clip end pileups so that the remainders of the histograms can reach full-scale. However, to minimize the possibility of misinterpretation, this option is not saved between sessions, so the default is always to display end pileups clearly. A text warning is also printed whenever an end pileup exceeds a configurable percentage of total events (default, 10%, e.g. line 39 Fig. 2). Since it is unclear whether end pileups include off-scale peaks, they are never reported as separate peaks (thus 2, not 3 peaks are reported for FL1 in Fig. 4).

```

MFI-generated 256 channel histograms
  for data from FCS file GDD49001:
Ungated. Total events in gate are
  5000 out of 5000 (100%)

Channel    FSC      SSC      FL1
0          0         0        929
1          0         0         1
2          0        214         1
3          0        438         1
4          0         77         2
...
56         99        229         0
57        109        264         0
58         95        233         0
...
69        210         49         0
70        230         59         0
71        183         39         0
...
135         4         0        580
136         4         2        676
137         2         0        594
...
254         1         0         0
255        22         1         0
  
```

Figure 5. Excerpts from ASCII file GDD49001.HIS produced by MFI (lines are omitted where dots are shown). MFI creates gated ASCII histogram data files for import into publication-quality graphics programs. In this instance, MFI was configured to reduce the 1024 channels in the list mode file to 256; it can be configured to display all 1024 channels, or to reduce the channels in factors of two between 512 and 16. The number of channels is reduced by summing adjacent channels, which provides a rudimentary form of smoothing. Savitzky-Golay smoothing is optional, and was not applied here.

Figure 4 was printed with the option to clip end pileups enabled. The left peak of SSC is very nearly zero in intensity, but is a real peak with two shoulders as can be seen in Fig. 5. (On request, MFI displays on the screen the event counts in the first and last five occupied channels to assist in interpreting the ends of a histogram.) Because the left SSC peak is real, the SSC histogram has been scaled to include it; this is why the peak to the right does not reach the top of the graph. (As can be seen in Fig. 5, the first SSC peak reaches 438 events, while the second, only 264.) The FL1 histogram includes an end pileup in channel 0 (the vertical line is part of the histogram; no Y axis is drawn; note the 929 events in channel 0 in Fig. 5). This has been clipped (the top of it is now off-scale) so that the 676 events in the peak of interest is at full-scale.

In the top panel of Fig. 6 are shown the FSC histograms for the standard bead sample in Fig. 4 (GDD49001) gated with the three gates shown in Fig. 4. Gate #1 includes single beads, gate #2 includes aggregates, and gate #3, air bubbles(?). To generate Fig. 6, the name of file GDD49001 was duplicated on the input file selection list with the '&' key. Gates #1-3 were then assigned, and histogram overlays 'H' requested as follows:

- 2H GDD49001
- 3H GDD49001
- 1 GDD49001

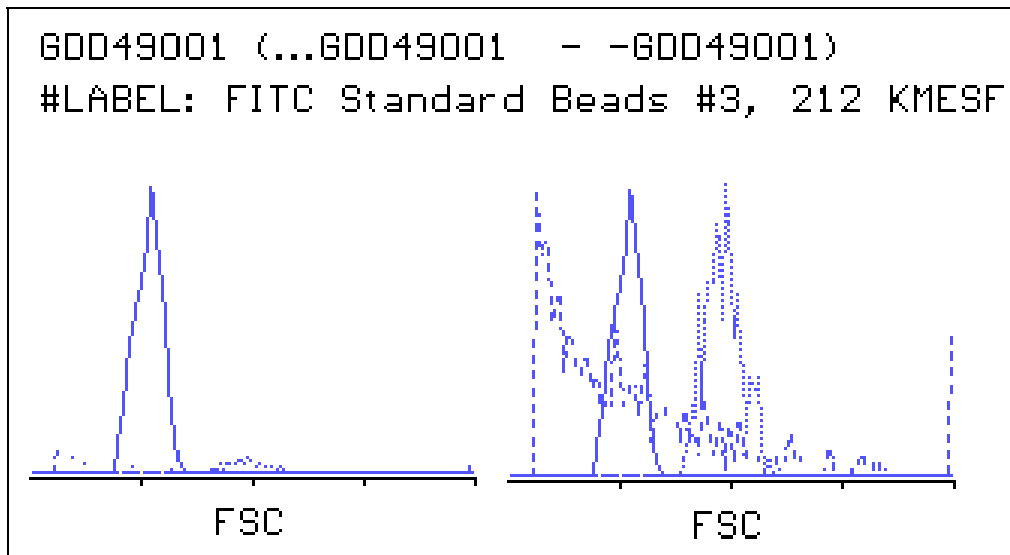


Figure 6. Scaling options for overlaid histograms. Magnified FSC histograms are overlaid for three different gates applied to the same list mode data file. The gates employed are shown in Fig. 4. In the left panel, all histograms share a common scale set to make the tallest peak reach the top of the panel (areas under peaks are proportional to events). In the right panel, the same data are displayed with each histogram scaled independently.

By default, MFI scales overlapping histograms on a common scale, so that the areas under each histogram are proportional to the numbers of events represented. This is seen in the left panel of Fig. 6; the peaks contain 19, 75, and 4.6% of the events. If one wishes to see small peaks more clearly at the expense of having areas under peaks unrelated to numbers of events, MFI offers the option of scaling overlaid histograms independently, as shown in the right panel of Fig. 6.

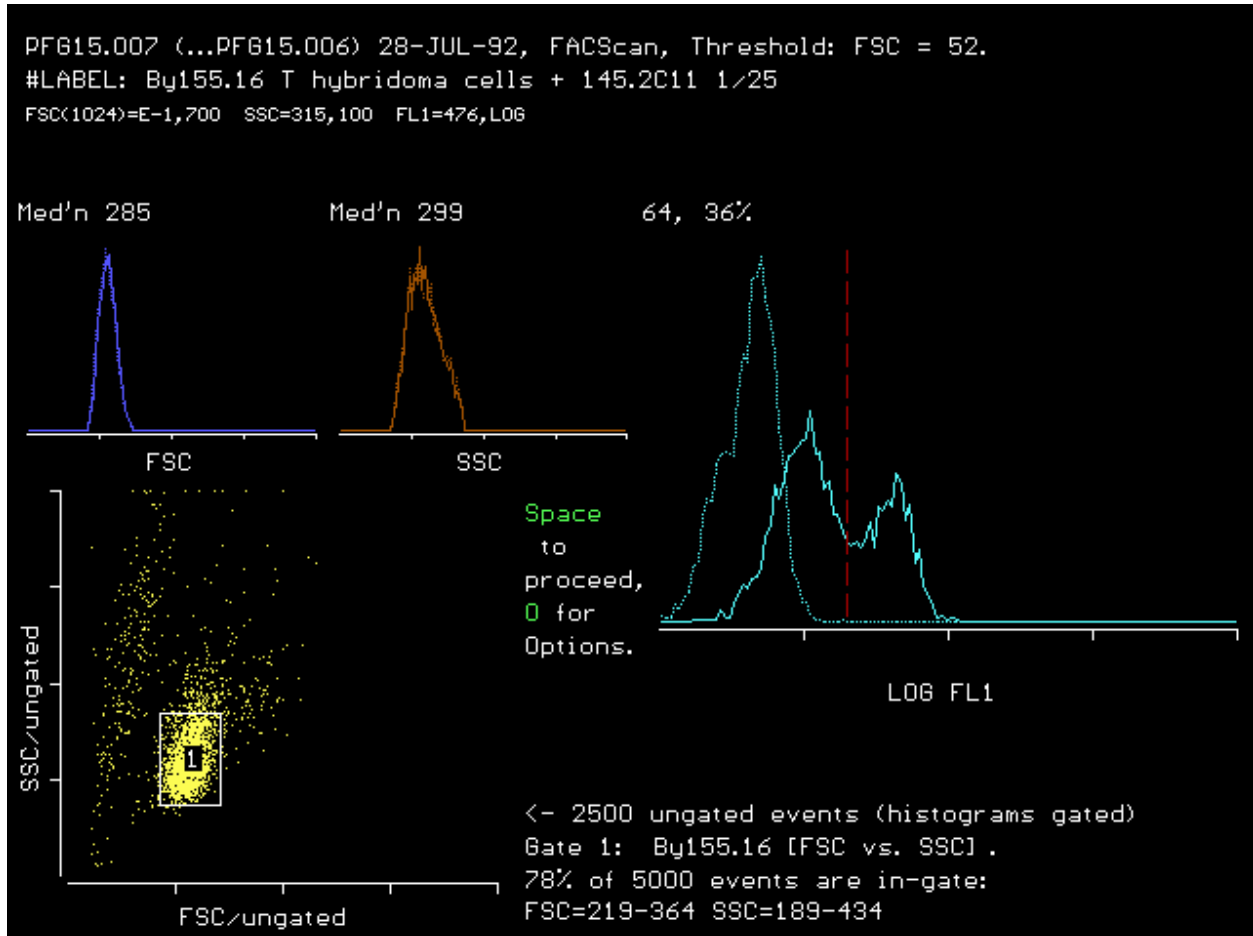


Figure 7. Automatic peak separation for heterogeneous T hybridoma cells stained indirectly for CD3. Dotted histograms are for unstained cells (autofluorescence). In the primary (text) output (not shown), the linear median intensities for the two FL1 peaks, corrected for autofluorescence, are reported to be 5 and 35.

Fig. 7 shows an example of automatic peak separation by MFI on a single-color sample of T hybridoma cells from a culture that was losing expression of CD3. MFI reports that 36% of the cells express CD3 at high density. When only 3 parameters are displayed, the third histogram (typically FL1) is enlarged. Fig. 8 shows a 3-color sample of human leukocytes stained for CD8, CD4, and CD3. Up to 6 histograms can be

shown simultaneously on MFI's summary graphics screen. Since all histograms in Fig. 8 are multimodal, only percentages are shown on the graphics screen; intensities for each peak are available in the primary (text) output (not shown).

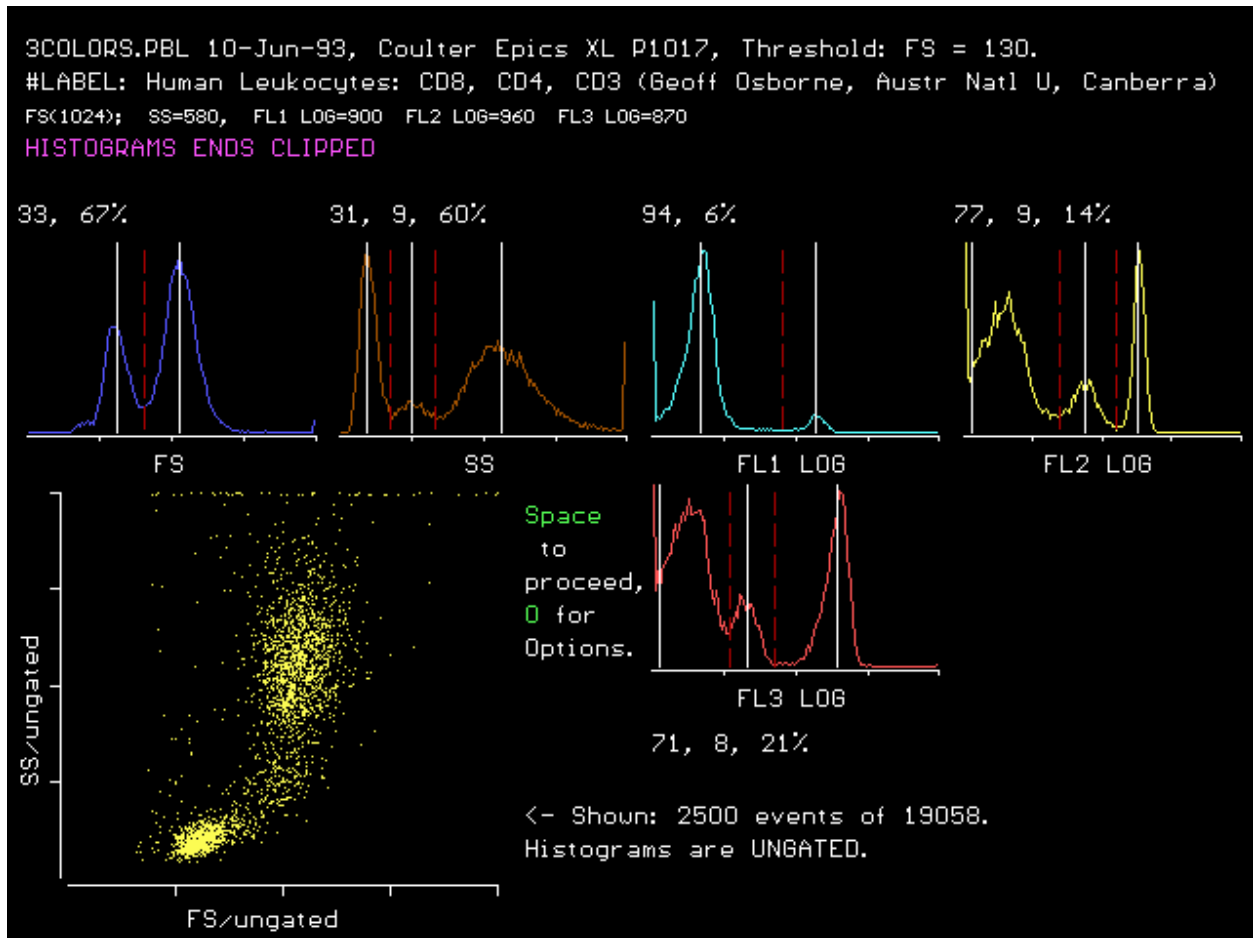


Figure 8. MFI's summary graphics screen showing 5 histograms and automatic peak detection on a 3-color sample of human leukocytes stained for CD8, CD4, and CD3 for FL1, FL2, and FL3 respectively. This data file was acquired on a Coulter Epics XL and was kindly provided by Geoffrey W. Osborne (John Curtin School of Medical Research, Australian National University, Canberra).

Time Slicing. MFI can divide a single continuously acquired list mode file into slices containing equal numbers of events. Scatter-gated median intensities are printed for each slice (not shown); optionally, a gated histogram ASCII data file is created for each slice (for use in producing publication-quality histograms). If the rate of events/second is constant during acquisition, the mean time of acquisition can be interpolated from the times when acquisition was begun and ended. The linearly

interpolated times for each slice are included in MFI's tabular output (not shown). When the cytometer records the acquisition time as a parameter for each event, the median or mean time for each slice is reported by MFI.

For the example of time-slicing shown in Fig. 9, FITC-conjugated monoclonal antibody was added to a sample of cells, the sample was vortexed, and immediately placed on the FACScan. Acquisition was delayed until the sample had flowed for 5 seconds to exclude the burst of spurious events resulting from the transient hyperpressurization the FACScan applies to the sample. Acquisition continued without interruption for 3.5 minutes, during which time 4780 events were acquired. The list mode file was divided by MFI into slices of 600 events each. The resulting slice intensities are plotted vs. time in Fig. 9.

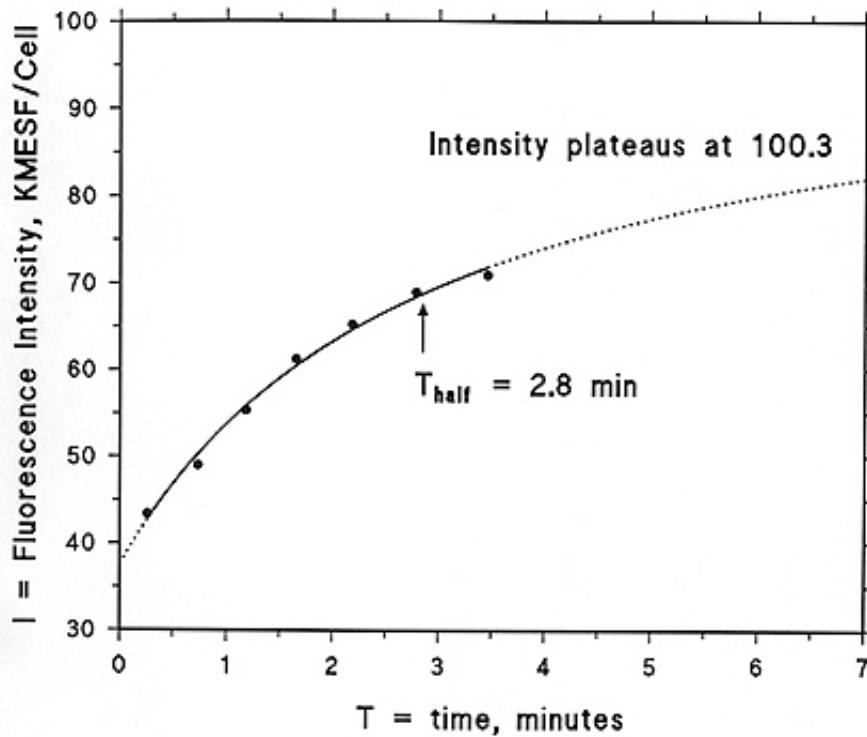


Figure 9. Results of time-slicing a single list-mode data file that recorded the binding of FITC-conjugated antibody to cells during acquisition. During acquisition, the live event rate display was recorded manually as a function of time after addition of antibody, using a stopwatch. There was a drop in the rate of about 30% by the end of acquisition, due to sedimentation of cells in the stationary sample tube. Rate vs. time observations were fitted with a 4th degree polynomial, and the resulting equation was used to estimate the mean time for each slice. The resulting data were fitted to a modified one-site binding Michaelis-Menton equation. The modifications include the fluorescence intensity of unbound antibody included in the core-stream volume, $I_{unbound}$, and the time on the

abscissa that, in the absence of vortexing, would have been required to account for the amount of antibody bound at time zero due to the accelerated binding occurring during sample vortexing, T_{vortex} . Thus, $I = (I_{\text{maximum}} ((T + T_{\text{vortex}})/(T_{\text{half}} + T + T_{\text{vortex}}))) + I_{\text{unbound}}$. Best fit occurred with $T_{\text{vortex}} = 0.08$ min and $I_{\text{unbound}} = 35.6$ KMESF. This figure was produced with the Fig P graphics and curve-fitting program.

DISCUSSION

MFI is efficient for batch processing of assays in which graphics need not be examined for every sample. It is also efficient when one wants to examine gated histograms for all parameters, and the scatter dot plot, for every sample, since it fits all of these onto a single screen, and one can move from file to file simply by pressing the Enter key. In most cases, the automatic peak detection works well, saving the time needed for manual placement of peak separation markers. Note that MFI does not require a color video monitor (although color is used when available), Microsoft Windows, nor a mouse. The present implementation has a number of limitations. Gates are limited to rectangular shapes, arbitrary separation lines cannot be placed manually on histograms, and mouse support is lacking. Quadrant or octant graphics and analysis needed for 2- or 3-color subpopulation work are beyond the intended scope of MFI; hence, MFI is most useful for single color work, or, if multicolor, for uniform cell lines.

The PECAN program written by Morgan P. Conrad and Ravi Mhatre while staff at Becton-Dickinson and placed in the public domain provides quadrant/octant capability for multi-color subpopulation work with a remarkably streamlined interface. PeCAN is available from flowcyt.cyto.purdue.edu. Source code is available both for MFI and for PECAN, and therefore interested parties are free to enhance or modify these programs as desired.

A much more elegant and highly developed program than PeCAN has recently been made available free of charge by the Salk Institute through the generosity of its author, Joseph Trotter. This program, WinMDI (Windows Multiple Document Interface for Flow Cytometry), which requires Microsoft Windows 3.1, complements MFI nicely in that it excels at graphics. It displays multiple gates in color on multiple dot plots, allows the setting of arbitrary regions on histograms, and is excellent for quadrant analysis, contour displays, and three-dimensional graphics. WinMDI allows the user to copy both text and graphics to the Windows Clipboard for pasting into other applications, including desktop publishing. It is as unsupported software from facs.scripps.edu.

ACKNOWLEDGEMENTS

MFI could not have been adapted to the broad range of cytometers that it supports, nor made available so effectively to the scientific community during its early development stages, without the Email Cytometry User's Group and anonymous ftp service generously made available by Stephen Kelley and J. Paul Robinson of the Purdue University Cytometry Laboratory. I am grateful to the National Science Foundation for grants that supported the acquisition of our FACScan and FACStar data and for their support of the Internet, to George Drake for providing the Biology Department anonymous ftp service at the University of Massachusetts, to Glenn D. Drabik for providing the results in Figs. 1-6, Peter F. Graves for providing the results in Fig. 7, Geoffrey W. Osborne for providing the results in Fig. 8, Dorie A. Barrera for providing the results in Fig. 9, to Dr. Wade Goolishian for donating standard beads, to Geoff Osborne for reporting numerous bugs during MFI development, and to Geoff Osborne, James Weaver, Stuart Gezelter, Eric Van Buren, and Nick Viner for providing sample data files. Mike Lance of Becton-Dickinson generously donated the IEEE-488 PC card used to transfer data files from our Hewlett-Packard computer to the Internet. Thanks are due Becton-Dickinson for contributing HP to PC IEEE-488 data transfer programs (HPPCLINK) to the public domain.

LITERATURE CITED

1. Dean PN, Bagwell CB, Lindmo T, Murphy RF, Salzman GC: Data file standard for flow cytometry. *Cytometry* 11:323-332, 1990.