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# Statistical Considerations in DNA Flow Cytometry

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*Abstract.* Flow cytometry is frequently used for assessing individual cell characteristic(s) for a large number of cells. It has a variety of medical applications including assessing the quantity of intracellular DNA and detecting the presence of antigens such as CD4. Flow cytometric variables are evaluated for their clinical prognostic value, particularly in cancer, and are often used for clinical screening of diseased patients. The prognostic worth of these variables is questionable and is controversial in the medical community. This controversy is caused in part by the multiple methods of analysis and the lack of adherence to quality control standards. The analysis of flow cytometric data presents a number of interesting statistical problems, particularly in deconvolution of overlapping distributions and detection of abnormal subpopulation(s) of cells. The current methods incorporate subjective procedures, may use ill-founded assumptions and yield differing results. This article summarizes the flow cytometry process of measurement and reviews unsolved statistical and quality control issues pertaining to the analysis of flow cytometric data. DNA histogram analysis is used to exemplify these issues.

*Key words and phrases:* Aneuploid; DNA; DNA histogram; diploid; deconvolution; fluorochromes; S-phase.

## 1. INTRODUCTION AND HISTORY

Flow cytometry presents a fertile research area for statisticians, particularly in deconvolution and estimation of overlapping distributions of unknown form. Presently there are multiple methods used in the field; most, if not all, of these methods incorporate subjective procedures. In addition, there are many quality control issues. This article is written with the intent of informing statisticians of the statistical problems inherent in modeling flow cytometry data and of the immense number of factors which influence the variability of the data.

Flow cytometry can be used on any particle (not just cells) and can be used to count a specific subset of particles, to sort a specific subset of particles away from the rest of the sample, and to measure a property (or properties) of each of the particles. Individual cells are isolated in a thin stream of fluid and then passed through an observation point where a property or characteristic of the cell is indirectly measured and then recorded. (The advantage of flow cytometry is the rapidity of the measurement, making it possible to measure thousands of cells over a short period of time. Image cytometry provides an alternative approach to the measurement of cell characteristics but is usually limited to a few hundred cells (review).) The flow cytometry process is described in more detail in Section 2.

Flow cytometry has many clinical applications. including hematological and immunological analyses, analysis of surface antigen expression (such as CD4 counts) and DNA content analysis in cancers. As pointed out by a reviewer, flow cytometry is currently used for screening a wide variety of clinical malignancies in cancer patients and patients with immune deficiency. "Clinical applications are also being pursued in the fields of genetics, microbiology, parasitology, pharmacology, reproductive biology and toxicology" (Shapiro, 1995, page 30).

The primary medical use of flow cytometry is the indirect measurement of intracellular DNA content.

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Measurement of the amount of DNA content in tumor cells gives an indication of cell proliferation, as well as cells with an abnormal amount of DNA, and thus may be of prognostic value in clinical cancer studies. This article presents statistical modeling problems that arise in analyzing DNA content in cells and discusses the magnitude of variability of flow cytometric measures. Some of the same issues arise in other applications of flow cytometry.

Immunologic phenotyping is probably the second most common application of flow cytometry. (An example of this is assessing the proportion of lymphocytes in an HIV patient that are CD4+.) Although the techniques used in immunology are different than those used in DNA analysis, both applications share statistical problems in discriminating between populations. A good critique on some of the statistical methods used for analyzing immunologic flow cytometry data can be found in Watson (1992). Bagwell (1996) presents an overview of the estimation methods used in flow cytometric immunofluorescence analysis.

The problems presented here may not be apparent to the statisticians who are recruited to analyze flow cytometry data. A quick glance at Medline shows 13 citations with flow cytometry in 1977; 915 citations between 1978 and 1982; 4,673 between 1983 and 1987; and 13,900 between 1988 and 1994. (A subsequent search shows 11,047 citations from 1991 through 1995.) An analogous search through the *Current Index of Statistics* yields less than a dozen citations which are directly pertinent to the analysis of flow cytometric measures. Hence, although the number of medical articles that use flow cytometric measures is increasing at a rapid rate, the small number of statistical articles that are directly applicable is somewhat discordant.

Dean and Jett published a landmark paper on DNA analysis in 1974, and since then hundreds of methods of modeling for DNA histograms have been proposed (Bagwell, 1993; Dean and Jett, 1974) (for a description of DNA histograms see below). These proposed methods, when published, are published in the flow cytometry literature and as a consequence have not come to the attention of most statisticians. In addition, the more modern flow cytometry instruments have built-in data analysis procedures that are frequently undocumented. I discuss popular modeling methods used in flow cytometry in Section 3.

In 1983 Hedley and co-workers (Hedley et al., 1983) described a method for measuring cellular DNA content using archival, formalin-fixed, paraffin-embedded tumors. This development, which made large retrospective studies possible, opened the door to the study of the relationship between flow cytometry measurements of cellular DNA content and long-term clinical outcome of patients. Since then there have been numerous studies of the prognostic value of these measurements in a number of different cancers. This paper will not address the prognostic value of the DNA content histogram variables; the literature on this is abundant and often not in agreement. The reader is referred to *Cytometry* **14**(5), for a report of the 1992 DNA Cytometry Consensus Conference (Hedley, Shankey and Wheeless, 1993) and the accompanying articles. In breast cancer, for example, many studies have found DNA measurements of prognostic worth (Beerman et al., 1990; Clark et al., 1989; Ferno et al., 1992; Gnant et al., 1992; Joensuu et al., 1992; Joensuu and Toikkanen, 1992; Kallioniemi et al., 1987; Lewis, 1990; Sigurdsson et al., 1990; Theissig et al., 1992; Toikkanen, Joensuu and Klemi, 1989; Uyterlinde et al., 1990; Witzig et al., 1991), while others (Dowle et al., 1987; Keyhani-Rofagha et al., 1990; O'Reilly et al., 1990; Ottestad et al., 1993) have found DNA measurements of questionable use. The disagreements within the literature may be due to the multiple methods of statistical modeling as well as the variability in the data and the lack of standardization for procedures. In their review article, Vindelov and Christensen (1990) state: "A review of the literature of the prognostic significance of DNA data reveals differences in the quality of the data and the statistical methods for deconvolution.... Data of this kind cannot be pooled to constitute a reliable basis for clinical decision making."

Flow cytometric measures have three major sources of variability: error due to processing of cells for the flow cytometer, instrument measurement error and inherent biological variation. In the next section I give a short overview of the process of flow cytometry, how it works, the goals of the process and, in particular, the methods used for analyzing DNA content in tumor cells. The main problem in modeling flow cytometric data is deconvolution of overlapping distributions. Section 3 presents modeling issues, a brief summary of the cell cycle, a critique of currently used models and other problems with currently used modeling methods including assessment of the quality of the data set. The tremendous amount of intermodel variability in flow cytometric variables is also discussed in Section 3. In Section 4 components of variability that are due to processing of the cells, due to measurement and due to different models are discussed in the context of quality control. Section 5 presents a brief conclusion.

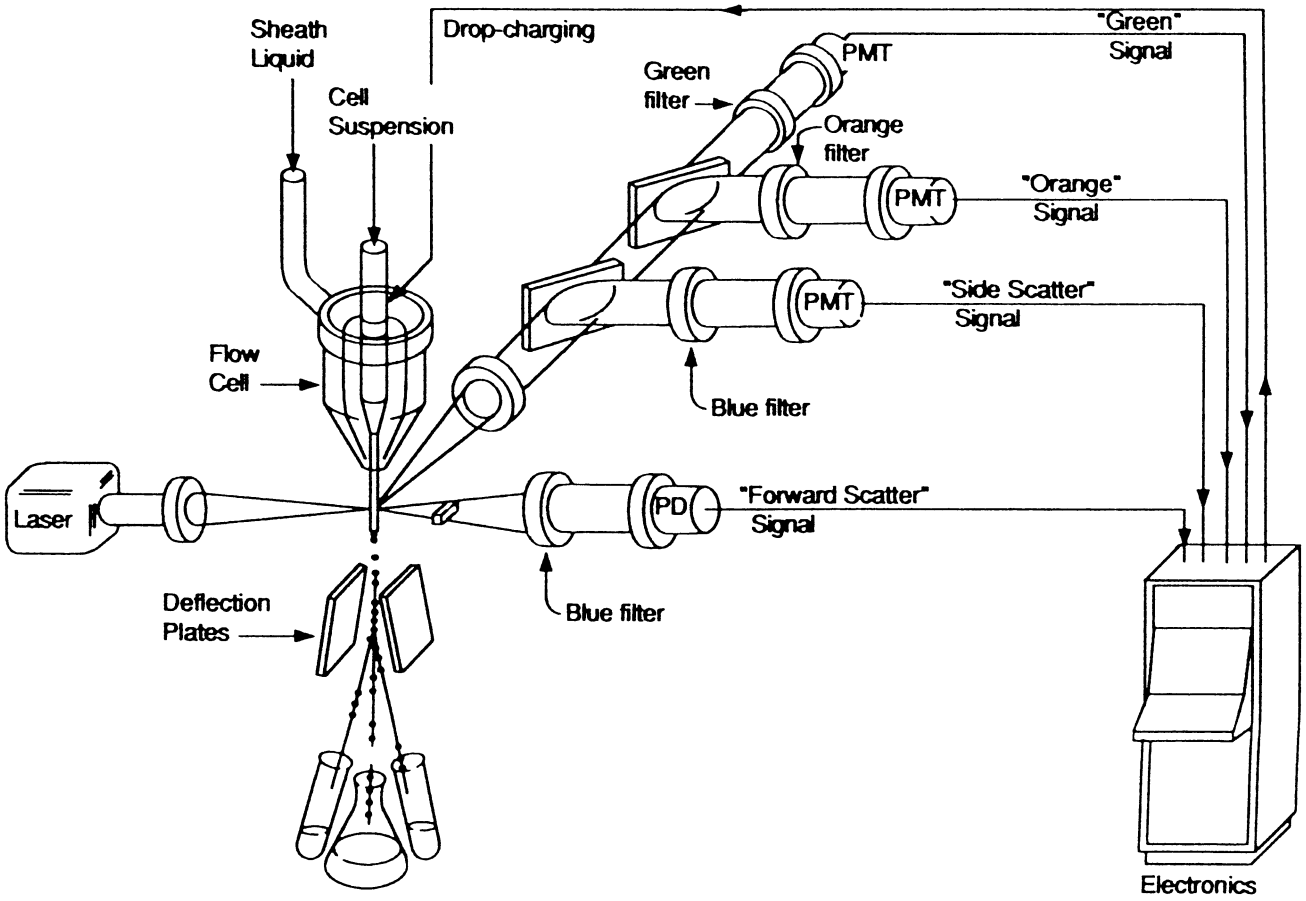


FIG. 1. *Components of a four-detector flow cytometer. Adapted by Givan (1992) from Becton Dickinson Immunocytometry Systems.*

## 2. THE PROCESS OF FLOW CYTOMETRY

Now I briefly describe a flow cytometer and how flow cytometry works. The interested reader is referred to Bauer, Duque and Shankey (1993), Givan (1992), Grogan and Collins (1990), Shapiro (1988, 1995), and Van Dilla, Dean, Laerum and Melamed (1985), among others, for more details on the process than are presented here. The reader whose primary interest is in statistical modeling issues can skip to Section 3.

### 2.1 The Flow Cytometer

Current flow cytometers are quite complex; Figure 1 shows the components of a flow cytometer in a somewhat simplified diagram. The flow cytometer sits on a stable surface, the optical bench, upon which a light source is fixed in rigid alignment with an observation point. A stained cell suspension is injected into the center of the flow chamber and passes through the observation point. Various fluorochromes are used to stain the particles, or cells, or cell characteristics, of interest. Once the stained cells have been illuminated they emit fluorescent light signals. These signals are detected by photo detectors and translated into an electric impulse the intensity of which should be proportional to the intensity of the emitted light signal. The intensity of this impulse is then digitized and recorded in a data bank. Referring to Figure 1 the reader will note four photodetectors: one for forward-scatter light, one for side scatter light, one for "orange" signal and one for "green" signal. With this flow cytometer, for each cell that passes through the observation point, up to four signals are recorded by the intensities of their corresponding electrical impulses. For example, if a cell were stained with two different fluorochromes (say, one emitting green, the other emitting orange), then four intensities would be recorded as this cell passes through the observation point: that of forward scatter, side scatter, green and orange. With current technology flow cytometers have up to six photodetectors.

### 2.2 The Histogram

After the data are collected, presentation of a histogram of the cell frequencies in each intensity class provides a summary. For each photodetector there can be a frequency histogram with a number of classes, usually either 256 classes (channels of light intensity) or 1,024 classes. A control sample can be used to define the histogram of light intensities that would be apparent from unstained cells. Figure 2 shows a univariate histogram from a control sample in the upper left-hand corner and then compares this histogram with samples that have been stained. When measuring DNA content in a cell the intensity signal is theoretically proportional to the amount of DNA in the cell. For each photodetector there is a univariate histogram of the frequency of cells per intensity class (channel). Bivariate plots can also be constructed from the readings of two photodetectors. Often these are presented as scatter diagrams, with each of the axes representing the intensity scale from one of the photodetectors, and the height of the histogram class is represented either by varying shades of gray to black, by color plots or by contour plots. Alternatively, a two-dimensional isometric representation of a three-dimensional histogram can be used. Reading measurements of the height of the histogram (frequency of cells) is somewhat difficult on bivariate plots.

## 3. DNA HISTOGRAM MODELING

### 3.1 The Cell Cycle and the Ideal DNA Histogram

The tumor cells are prepared, isolated, stained (with a stain specific for DNA) and injected into the flow cytometer for analysis. Ideally, the stained cells fluoresce with an intensity proportional to the cellular content of DNA. Essentially, the intensity of the signal from each photodetector is summarized in a univariate histogram or from two photodetectors in a bivariate plot (details of this process are described in Section 3.2).

Examples of DNA histograms from a recent article (Isobe et al., 1995) are shown in Figure 3 (definitions of terms in the caption are in Sections 3.4 and 3.5). To understand the components of this graph, a brief review of cell biology is needed. After discussing the DNA histogram of normal cells, I discuss the abnormalities that frequently occur with tumor cells.

With the exception of germ cells and cells preparing for division (mitosis), all healthy somatic cells in an organism have the same amount of DNA. Since each normal somatic cell contains two copies of $N$ chromosomes this quantity of DNA is referred to as $2N$ or $2C$ and is called diploid (Omerod, 1994; Givan, 1992). In healthy tissue a large majority of the cells will have $2N$ DNA (this phase is called $G_1$, a resting phase is called $G_0$). A smaller proportion of healthy cells will be in the process of synthesizing DNA for cell division; hence these cells have between $2N$ and $4N$ DNA. This period of cell life is referred to as S-phase. Once a cell has twice its normal DNA content ($4N$, in the phase called $G_2$) it makes the final preparations for cell division and then divides into two cells (a phase called $M$ for mitosis) each with $2N$ DNA.

The perfect DNA histogram of normal cells would consist of a spike at a signal intensity that would come from cells with $2N$ DNA ($G_1/G_0$, or diploid), a spike of less than half the height of the diploid spike at twice that intensity ($G_2/M$ cells) and a distribution shorter than the $G_2/M$ spike ranging over the intensities in between from cells in S-phase (Figure 4b). Due mostly to the spectral distribution of the fluorochrome and the lack of a perfect one-to-one correspondence between the DNA and the fluorochrome present, as well as the other sources of variability (refer to Section 4), the ideal DNA histogram is just that, an unrealizable ideal. As a consequence the histogram that results is a distribution of intensity signals which theoretically has a mode centered over the intensity for diploid cells and a shorter mode centered over the intensity for $G_2/M$ cells (see Figure 4). A reviewer points out that in perturbed samples of cells the mode over the intensity channel for the $G_2/M$ cells may be higher on the frequency scale than the mode for the $G_1/G_0$ cells.

### 3.2 Analog-to-Digital Conversion: Why a DNA Histogram Is a Data Set

The intensity of the signal from each photodetector is translated to an electrical impulse and amplified either through a linear function or a logarithmic function. Flow cytometers have an analog-to-digital converter (ADC) which converts the amplified electrical impulse (a continuous variable) to an integer channel number (a discrete variable). The resolution of the ADC dictates whether there are 256 channels (0 to 255) or 1,024 channels (0 to 1,023) and consequently defines the resolution of the histogram. By digitizing the amplified signal from the fluorescing dye the ADC is creating the DNA histogram; so to flow cytometrists a DNA data set from a tissue sample and its corresponding DNA histogram are somewhat analogous. The continuous measurement from the amplified signal is not retrievable.

A few observations are of import here. The typical problem with histograms exists here in that

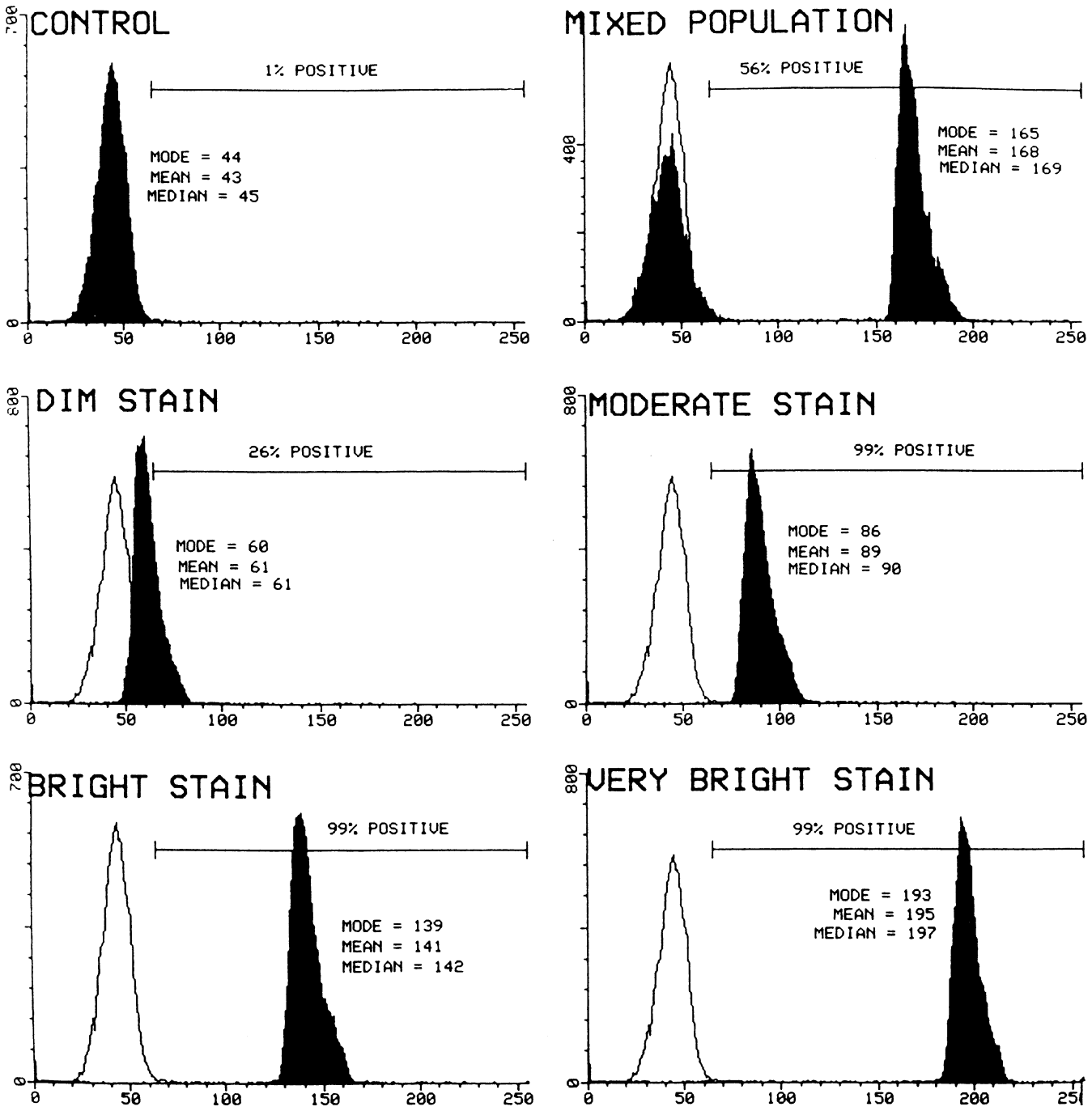


FIG. 2. *Histograms comparing control populations with stained populations and different brightness of stain from Givan (1992): vertical scale represents the number of cells within an intensity channel, or bin. On the horizontal scale are the channel numbers (proportional to measured fluorescence intensity); here 256 channels are used.*

too few channels may not provide enough detail to preserve the accuracy of the original analog signal. With a higher-resolution ADC (1,024 channels), DNA Cytometry Consensus Conference Guidelines (Shankey et al., 1993) recommend a minimum of 10,000 cells to insure that minor statistical fluctuations in the data set are not seen as a measurable attribute. Note also that an increase of 10% in analog signal is needed to jump from channel 10 to channel 11, but an increase of 0.1% in signal is needed for the jump from channel 1,000 to 1,001. Hence the higher the channel number, the higher

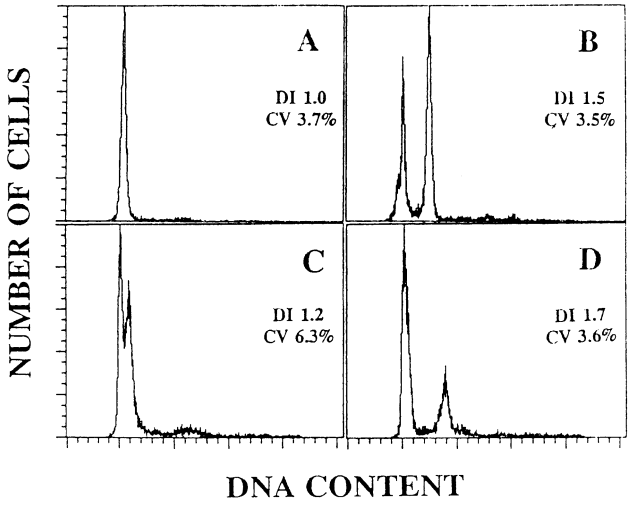


FIG. 3. *Representative DNA distribution histograms prepared from mesothelioma paraffin blocks*: *histogram A, diploid*; *histogram B–D, aneuploid. Histograms C and D were obtained from two different paraffin-embedded blocks of the same tumor* (*DI, DNA index*; *CV, coefficient of variation*). *From Isobe et al.* (*1995*).

the resolution of the DNA histogram. Watson (1992) points out that this introduces a positive skew into the recorded histogram. A cytometrist can shift the histogram into the channels with higher resolution (hence decreasing the band width of the histogram channels) by increasing the detector voltage (review; Shapiro, 1995).

### 3.3 Modeling of the DNA Histogram

Let $X$ represent DNA content of a cell. Let $Y$ represent signal intensity (i.e., channel intensity) of a single observation from a flow cytometer. Then $Y = X + \varepsilon$, where $\varepsilon$ is error due to measurement and due to processing of the cell. The goal of DNA histogram analysis is estimation of the distribution of $X$. In normal cells this distribution has three components: cells in $G_0/G_1$, cells in S-phase and cells in $G_2/M$. The common assumption about the density of $\varepsilon$ is that it is Gaussian. The main problem here, in estimating the distribution of DNA content for a sample of cells, is one of deconvolution.

The percentage of cells in S-phase (S-phase fraction, or SPF) is the measurement which is used as a proxy for tumor proliferation and may be of clinical prognostic value. Estimating SPF necessitates modeling the DNA histogram to estimate the distribution of $G_1/G_0$ (or diploid cells), that of S-phase cells and that of $G_2/M$. A critique of the methods used currently is summarized below for normal cells; the situation becomes more complicated when analyzing tumor cells (Section 3.4).

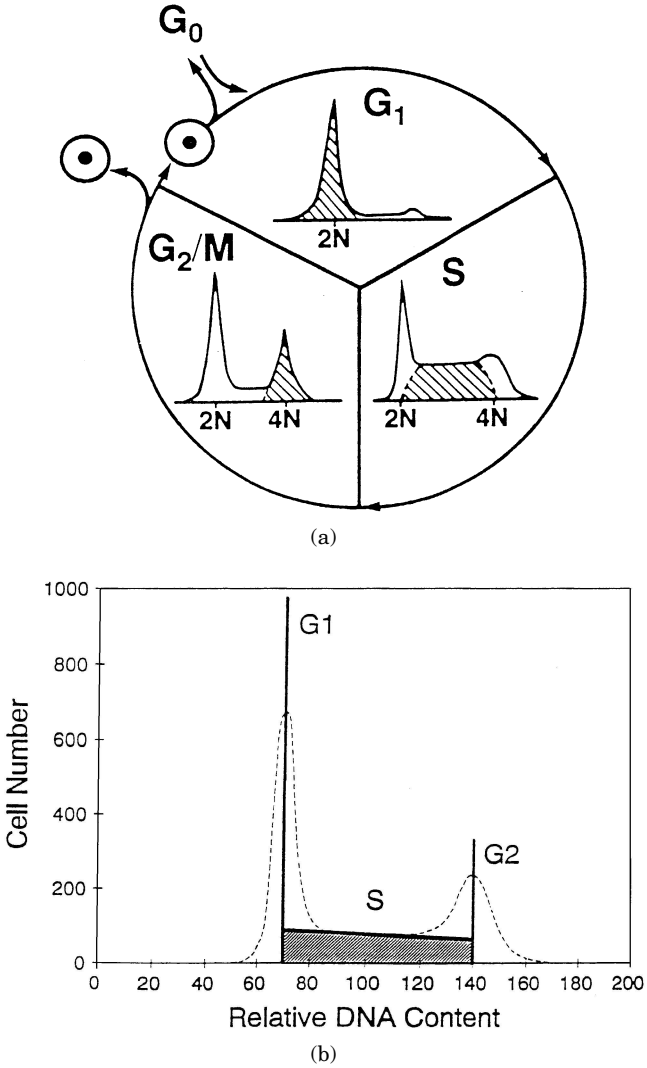


FIG. 4. (a) *The cell cycle and the components of the DNA histogram*: $G_0$ *and* $G_1$ *have* $2N$ *component of DNA*; *S-phase has between* $2N$ *and* $4N$ *component of DNA*; *and* $G_2/M$ *has* $4N$ *component of DNA. From Rabinovitch* (*1993*). (b) *A "perfect" DNA histogram for healthy cells versus an idealized empirical DNA histogram. The solid lines represent a theoretical representation of a DNA histogram if there were no errors in measurement*: *the spikes represent the fluorescence of the DNA from cells in* $G_0/G_1$ *and* $G_2$ *respectively*; *the trapezoid in between the spikes represents the distribution of fluorescence from DNA from cells reproducing DNA in S-phase. The dotted lines represent the theoretical histogram of fluorescence from DNA with errors in measurement which assumes that the error component results in a Gaussian broadening of the peaks. From Rabinovitch* (*1993*).

Presently it is assumed that the distributions of normal cells in $G_1/G_0$ phase and those in $G_2/M$ phase are symmetrical about their respective modes and are Gaussian. These assumptions arise from assuming that $\varepsilon$, the error per intensity channel, is Gaussian. Most, if not all, of the approaches to mod-

eling DNA histograms make these assumptions. As there is no "gold standard" for human cells it is not known if these assumptions are plausible. The left side of the $G_1/G_0$ phase and the right side of the $G_2/M$ phase are observed, but the other sides of these distributions are obscured with the S-phase distribution. Bagwell (1993), Dean (1985) and Rabinovitch and Jacobberger (1995) give expositions of the more commonly used procedures. Mendelsohn and Rice (1982) proposed a method of deconvolution using B-splines assuming a constant coefficient of variation across intensity channels. Using maximum likelihood, Vindelov and Christensen (1990) use a mixture model to fit DNA for deconvolution.

The assumptions about the shape of the S-phase distribution are less rigid. A simplified method makes no attempt to model this distribution but rather estimates the percentage of cells in S-phase as the remainder of the histogram after subtracting the two "Gaussian" components. This "outside-in" method is very sensitive to skewness in the two Gaussians (since it assumes no skew) and tends to underestimate S-phase (Bagwell, 1993). Alternatively, the S-phase distribution is modeled in a variety of ways, including extrapolated rectangular, extrapolated trapezoidal and extrapolated polynomial. In each of these "extrapolated" models the S-phase region is defined, the model (e.g., rectangular) is fit to the DNA histogram in this region and then extrapolated to the modes of the $G_1/G_0$ phase and $G_2/M$ phase (Bagwell 1993). Fried (1976) proposed using a series of broadened Gaussian curves to fit the S-phase distribution. Vindelov and Christensen (1990) assume that "S-phase distribution can be described by the exponential function of a polynomial of a given degree."

The problem becomes even more complicated by the presence of readings from more than one cell (aggregation) and readings from debris in the sample. Readings due to aggregation and debris need to be either screened out or accounted for in the model. Estimation of the part of the histogram which is attributable to debris and aggregate cells is also an unsettled problem. The debris is modeled assuming that any signal intensity below that covered by the distribution of the diploid cells is debris (and yet this may be due to cells with a deficient amount of DNA). The shape of the distribution of debris is assumed to be exponential, or sometimes truncated exponential (Vindelov and Christensen, 1990), when the modeling is parametric. Nonparametric approaches, which are histogram dependent, assume that the distribution of intensities from debris would behave as intensities emitted from sliced nuclei. These latter approaches seem to fit better, as the exponential model tends to overestimate the component of debris present past the $G_1/G_0$ peak (Bagwell, 1993; Rabinovitch, 1993; Shankey, et al., 1993)

There are two popular ways to account for readings from aggregate cells; both are ad hoc. One approach is to gate out (exclude) readings from aggregate cells as they pass through the laser beam. If there is a dip in the pulse, then it is likely that this pulse has come from two cells in quick succession to one another. However, this method is not sensitive to aggregate cells which are clumped such that one cell is behind the other with respect to the laser beam; such a double cell would not be distinguishable from a single cell with $4N$. In addition, cells which are heterogeneous in shape (not aggregate) may produce a pulse with a dip.

The other approach is to model the component of the histogram that is due to aggregate readings. Usually, the component of the distribution which is assigned to be aggregate is based on the distribution beyond the distribution of the tetraploid cells. Current models for aggregation arise from attempts to model the probability distribution of aggregation (including doublets, triplets, quadruplets etc.) as a function of surface area of the cells (or nuclei) and a constant probability of aggregation (Bagwell 1993; Rabinovitch, 1993). However, it is also possible that readings beyond the normal tetraploid range are an indication of cells with an abnormal amount of DNA.

A third approach, pointed out by a reviewer, is software gating which excludes observations from the data list. In this method the data are listed in a multivariate format and can be displayed in histogram(s), and the flow cytometrist can set gates based on values determined from the histogram(s). Wheeless (1991) gives an example of software gating (in a data set with variables nuclear fluorescence, nuclear diameter and cell diameter) where the gate is defined to subset the data list to those observations with a low ratio of nuclear diameter to cell diameter. In DNA analysis the cytometrist can set a gate based on the peak fluorescence intensity and integrated intensity (reviewer) to screen out aggregate cells.

Whether aggregate cells should be gated out (through hardware, described first, or software, described third) or modeled is controversial (Shankey et al., 1993). Modeling aggregation is probably more reliable than attempts at gating out intensities from cells with bimodal pulses as the latter will not be sensitive to cells that pass through the laser beam in a formation which is parallel to that of the beam or in a very tight formation. All methods

share the difficulty of not using objective criteria for screening out aggregate readings. It should be noted that using both gating and modeling for aggregation will over compensate (Bagwell, 1993; Shankey et al., 1993).

### 3.4 Modeling of DNA Histograms from Samples of Cancer Cells

I now turn to the modeling of tumor cells. There are two main questions to address in DNA analysis of tumor cells. The first of these is: "Within a tumor, are there more cells proliferating than would be expected in healthy tissue?" One measurement that has been proposed as a quantification of cell proliferation is the percentage of cells that are in S-phase. The existence of subpopulation(s) complicates the modeling problems into one of deconvolution of multiple subpopulations including quantifying the S-phase fraction for the normal cells and for the abnormal cells (see Figure 3, parts B and C).

In addition to the modeling issues discussed previously clinicians find a need to categorize the percentage of cells in S-phase into possible risk groups. A categorization of "high S-phase" is thought to be an indication of high cell proliferation and is used as an explanatory variable for clinical outcome of patients. A difficulty arises in defining high S-phase, since (1) methods for defining the distribution of S-phase cells are multiple and subjective, and (2) there is much variability in the flow cytometry data itself. Even the definition of high S-phase is very subjective: Shankey et al. (1993) suggest the use of "high S-phase," "intermediate S-phase," and "low S-phase" as a preferable categorization and, further, that these should be defined within laboratory and within tumor type. A recent advertisement for a DNA analysis software package emphasizes the subjectivity of cutoffs between "high," "intermediate," and "low" S-phase by stating "You've determined *your* lab's S-phase cutoffs, so where does *this* analysis fit into those ranges. Enter your cutoffs . . . and it happily answers the question and more" (Verity Software House, Inc., 1996). Often data-driven "cut-point" analyses are used to define the cut-point between "low S-phase" and "high S-phase," giving a researcher the false impression that there is an objective statistically significant difference between the clinical outcomes of the "low S-phase" group and the "high S-phase" group.

Another primary goal of analyzing DNA histograms from tumor cells is to answer the question: "Are there cells which have an unusual amount of DNA?" The existence of one or more subpopulations of cells with an unusual amount of DNA may be prognostic of clinical outcome. There are two statistical issues here. The first problem is finding an objective method for detecting whether such a subpopulation exists. The second issue is that presence of such a subpopulation complicates the modeling issue presented in the previous section into one involving the estimation of overlapping distributions from multiple subpopulations.

Cells which are neither diploid, nor diploid cells that have doubled their DNA for mitosis, nor diploid cells synthesizing DNA are referred to as *aneuploid* cells. There are two definitions of aneuploidy; the distinction is not often made in the literature but it is an important one. Cells with an abnormal number of chromosomes (not $2N$ in $G_0/G_1$) are called karyotypic aneuploid; this cannot be observed with DNA flow cytometry data described here. DNA-aneuploid cells have an unusual DNA content in their $G_1/G_0$ phase that is replicated through S-phase and into the $G_2/M$ phase; their presence is unusual in healthy cells. Although many tumor cells are DNA diploid, a tumor cell may (more commonly) develop extra DNA or may be missing some of its initial DNA. If there are a number of tumor cells which are DNA-aneuploid, a peak will be formed on the DNA histogram which is neither a diploid peak nor a tetraploid peak, but an aneuploid peak. Distributions of aneuploid peaks have been developed from rat tumor cells, and the development of a peak can be seen in Figure 5. In this figure the aneuploid peak is evident only five days after implantation of prostatic carcinoma, and the subsequent histograms give strong evidence that this is an aneuploid peak rather than an artifact of sample preparation or variability in the data (Section 4).

When modeling DNA histograms of human tumor cells the cytometrist does not have a series of histograms over time. Aneuploid peaks can be difficult to discern from statistical noise in the S-phase region and, conversely, peaks which are thought to be aneuploid could be due to variability in the data (see Section 4). Cusick, Milton and Ewen (1990) discuss the problem of discerning an aneuploid peak created from a small proportion of aneuploid cells or where the aneuploid peak is close to the diploid peak. Sometimes another variable can be measured to distinguish between aneuploid cells and diploid cells. For example, Gong, Traganos and Darzynkiewicz (1993) used cyclin B (protein expressed during $G_2$) as a marker to distinguish between diploid cells in $G_2$ and aneuploid cells with around $4N$ content. However, most of the methods used for discerning whether a subpopulation of aneuploid cells exists are totally ad hoc, often

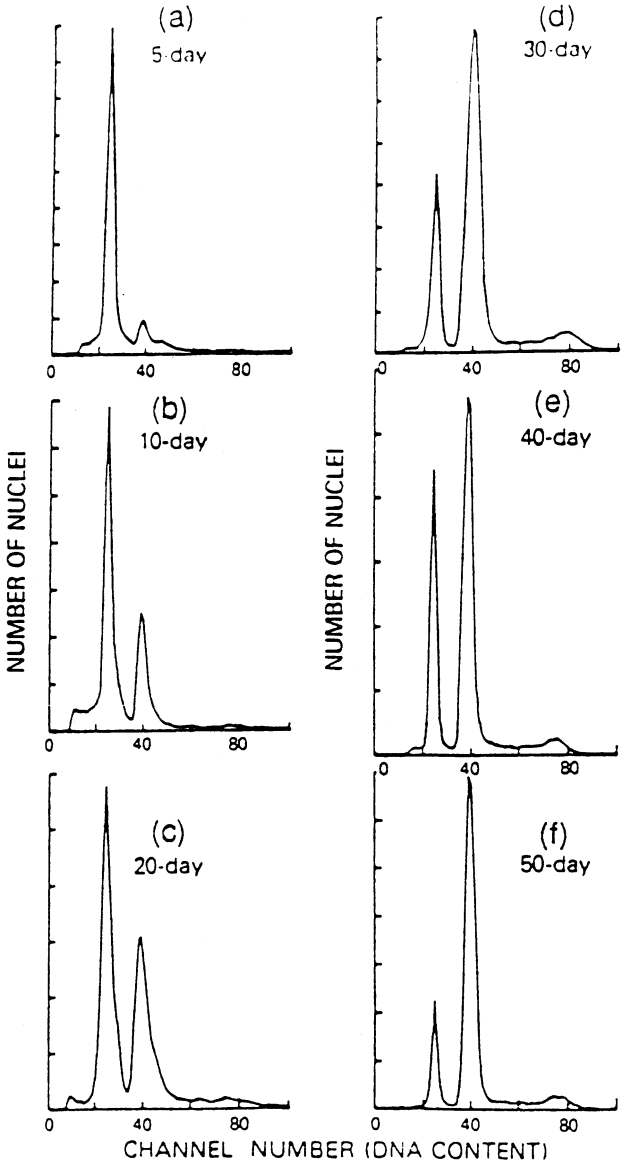


FIG. 5. *Increase in aneuploidy of rat prostatic carcinoma with age: the DNA distribution of an* R 3327 G *carcinoma* 5–50 *days after implantation, from Grogan and Collins (1990) reprinted from Collins et al. (1981). In panel* (a) *the peak at channel* 25 *is from diploid cells* ($2N$); *the peak at channel* 40 (*DNA index* $40/25 = 1.6$) *is from aneuploid cells. The proportion of aneuploid cells is seen to increase with time from implantation of tumor.*

nothing more than a subjective call from the flow cytometrist. Even when a peak looks suspicious for aneuploidy, it is difficult to discern the fraction of cells that are aneuploid as the distribution overlaps with the diploid cells' distribution. The question also arises of how to distinguish the component of the distribution which is due to aggregate cells from the contribution due to cells with an abnormally high content of DNA that are at the end of their cell cycle.

Using a DNA diploid reference, or control sample, can help to identify the diploid peak in the tumor sample and thus help with the detection of an aneuploid peak and the estimation of S-phase (Dressler, 1990; Muirhead, 1993; Rabinovitch, 1993; Shankey et al., 1993). Ideally the control sample is nonmalignant epithelial tissue from the same site and patient which has been handled identically to the tumor sample. All of these authors point out that in paraffin-embedded tissue a suitable control sample (which should also be paraffin-embedded) is usually not available. Thus detection of aneuploid peaks can be quite difficult and is subjective and nonverifiable.

Even when an aneuploid component is clearly present, if the aneuploid peak is close to the diploid peak, it creates problems in analyzing the histogram. Since the peak is overlapping the S-phase region for the diploid cells it is impossible to distinguish the S-phase fraction of diploid cells from the distribution of aneuploid cells and their respective aneuploid S-phase distribution.

Another variable which is commonly studied for prognostic significance (review) is the DNA index (DI). The DNA index is the ratio of the modal $G_1$ fluorescence channel of aneuploid cells to that of diploid cells. Hence the determination of the existence of an aneuploid peak and the positions of the $G_1$ peak for the diploid and the aneuploid population becomes important.

### 3.5 Model Fitting and Assessing Data Quality

Open statistical problems also exist in fitting models and assessing the quality of the data. Measurement of the two primary variables, percentage of cells in S-phase and presence of aneuploid subpopulations, rests heavily on the model used to analyze the DNA histogram and on the quality of the data.

Choosing which method is appropriate for each histogram is, at present, a subjective call. Van Dilla (1985) and Dean (1985) give a short summary of how to choose a method based on the shape of the histogram itself. Van Dilla goes on to point out that when cell growth is perturbed (through chemotherapy) the S-phase distribution may be altered in such a way as to make modeling difficult, resulting in inaccurate estimates. As there is no direct way to measure S-phase fraction in paraffin-embedded samples it is not known how accurate any of the models are even in analyzing samples of healthy cells. Baisch et al. (1982), using both simulated data and experimental data, compared 12 different methods of modeling DNA histograms. These authors found that goodness-of-fit was not a criterion for accuracy of S-phase fraction estimate because models with sim-

ilar goodness-of-fit statistics can yield dramatically different estimates for the fraction of cells in S-phase. They also found that variations in the shape of the DNA histogram in the S-phase region affected the accuracy of S-phase fraction.

Currently, models are fit by assessing a $\chi^2$-statistic. As the models are convolutions of different components, a nonlinear least squares algorithm is used to minimize the $\chi^2$-statistic. The method that Bagwell cites as being most frequently used is that of Marquardt (1963). This method finds the minimum $\chi^2$-statistic by combining two search methods. The first is a gradient search method which estimates the direction of maximum in the $\chi^2$-statistic as a function of the model parameters and changes the estimates to minimize the statistic. The second search minimizes the first-order Taylor expansion of the $\chi^2$-function (for the current model) with respect to the model parameters. The algorithm varies in choosing the optimal method of search at the current set of parameter estimates. The rate of this variation and the starting point can be adjusted to suit the needs of DNA histogram modeling. The algorithm also gives error estimation for the model parameter estimates.

The quality of the data set forming the DNA histogram is often assessed by estimating the coefficient of variation (CV) for the $G_0/G_1$ peak and the CV of the $G_2/M$ peak. Yet again there are different methods to do this, some of which are dependent on a flow cytometrist graphically defining the peak in question. These ad hoc calculations are based on the assumption that the underlying peaks are Gaussian. Even deciding which data sets (presented as histograms) are "clean" enough for analysis is a subjective call. Shankey et al. (1993) suggest guidelines for determining which DNA histograms are adequate for detection of an aneuploid population and for estimation of S-phase. Benson and Braylan (1994) give guidelines for CV's of peaks, proportion of cells within peaks and separation of peaks (DNA index of aneuploid peak) for detection of aneuploidy.

### 3.6 Intermodel Variability of DNA Histogram Variables

Silvestrini and the SICCAB Group for Quality Control of Cell Kinetic Determinations (Silvestrini, 1994) found tremendous intermodel variation in S-phase estimates on breast cancer tissue when the laboratories were under strict quality control (review). They compared four different models and found the intermodel discrepancies were more apparent in aneuploid tumors. Weaver et al. (1990) found that the correlation between measurements from frozen tissue and those from paraffin-embedded tissues differs with algorithm for removal of debris component from the histogram. Frierson (1991) and Herman (1992) also observed a high reproducibility of S-phase estimates between institutions when the same method of modeling was used, but the estimates differed when different methods of modeling were used. These intermodel differences in S-phase estimates were not significant when healthy tissue was analyzed. However, results between laboratories are frequently not consistent. Hitchcock (1991) found variability between methods of modeling the same data and also between laboratories using the same method of modeling. Kallioniemi et al. (1994) describe an algorithm for fully automated DNA histogram analysis, measuring DNA index, S-phase fraction and presence of an aneuploid peak, that they found works rather well and is consistent with conventional DNA histogram analysis. Kallioniemi, Joensuu, Klemi and Koivula (1990) observed interlaboratory variation in detecting aneuploid populations, DI and S-phase estimation within the same method of modeling. Discordant ploidy and DI status led to differing estimation of S-phase. In 4 of the 33 paraffin-embedded tissues two experienced laboratories disagreed on ploidy. For the other 29 cases the correlation between the S-phase estimates was quite high ($r = 0.90$). Baldetorp et al. (1995) found discrepancies across 12 laboratories due to differing categorizations of S-phase, differing methods for detecting aneuploid populations and differing estimators for S-phase fraction. These authors state that "the most suitable method(s) for SPF calculation remain(s) to be established."

### 3.7 Summary of Modeling Issues in DNA Histograms

In summary, choice of method of DNA histogram analysis can have a dramatic effect on the value of the resulting variables. Many of the procedures for analyzing the histogram (choice of model, detecting presence of aneuploid population, measurement of quality of the histogram) are ad hoc. In order for the flow cytometric variables to be of scientific use there must be methods of modeling which are more objective and based on valid statistical models. Currently, the validity of the models being used is questionable, as is the objectivity of some of the measurements. There are a wealth of statistical problems to be solved which include the following:

1. estimation of overlapping distributions ($G_0/G_1$ phase, S-phase, $G_2/M$ phase, debris distribution, aggregation distribution);

2. quantitative recognition of the existence of abnormal subpopulations and estimation of their overlapping distributions;
3. estimation of the variability of flow cytometric variables that result from (1) and (2);
4. estimation of the variability in flow cytometric variables due to different modeling techniques, different laboratory processes, and so on;
5. quantification of model fit that picks the model that yields the most accurate flow cytometric measurements (S-phase fraction, presence of aneuploid population(s)).

In addition, a reviewer points out that analysis of multivariate flow cytometric measurement (measurement from more than one characteristic of the cell) "will be a fertile research arena." For example, the bivariate measure of DNA content and bromodeoxyuridine (BrdUrd) is being studied for the measurement of tumor potential doubling time (Begg, 1995; Terry and Peters, 1995). Cells can be pulsed in vivo with BrdUrd or another thymidine analog. Those cells that are replicating DNA will replace thymidine with BrdUrd. The sample can then be stained with fluorochrome-tagged monoclonal antibodies specific for BrdUrd and fluorochromes specific for DNA to produce the bivariate response (Darzynkiewicz, 1993). Vindelov and Christensen (1990) emphasize the need to use correlated bivariate analysis for deconvolution of two or more overlapping populations within the S-phase region. In addition, dual-parameter (bivariate) flow cytometry is currently being used for estimation of the proportion of cells in resting phase ($G_0$) (Pellicciari et al., 1995).

## 4. QUALITY CONTROL

Variability in flow cytometric data arises both from the preparation of the cells, from the process of measurement itself and from the interaction of both of these. Quality control issues are readily apparent. Standards and guidelines are under continual review by professional societies (International Society of Analytical Cytometry, Society of Clinical Cytometry) and are presented at workshops of conferences (Rabinovitch and Jacobberger, 1995); several recent articles (Dressler, 1990; Muirhead, 1993; Shankey et al., 1993; Silvestrini, 1994) summarize the problems with the inconsistencies in laboratory process and suggest guidelines. Lack of adherence to suggested guidelines is common and, as Shapiro (1995) points out, many operators of flow cytometers have insufficient training. The result is that flow cytometry data is often quite "dirty" and this has a direct bearing on the subsequent analysis of the data. Some of the major sources of variability, and their influence on DNA data and histograms, are summarized in this section.

To start with, the intensity of light emitted by the fluorochromes is not necessarily proportional to the presence of the quantity being measured (here, DNA). This can result from the type of fluorochrome being used, the preparation of the cell suspension, the orientation of the cell suspension within the flow cytometer, as well as the inability of the fluorochrome to behave as expected. Rabinovitch (1993) states that only a portion of the DNA is accessible to binding and, further, that this accessibility is dependent on "cell type, cell cycle status, cell differentiation, and cell viability." Additionally, staining and fixation have an interactive effect on the accessibility of the DNA binding sites (Becker and Mikel, 1990; Darzynkiewicz, 1990; Dressler, 1990; Rabinovitch, 1993; Larsen, Munch-Peterson, Christiansen and Jorgensen, 1986). Kusuda and Melamed (1994) report that there are time-dependent changes in fluorescence caused by changes in the equilibrium of the dye solution, changes in the flow stream, cell settling, changes in permeabilization, denaturation and other artifacts. Several authors find that different staining protocols can lead to differences in DNA measurements (Hitchcock and Ensley, 1993; Rabinovitch, 1993). Shankey et al. (1993) maintain that it is critical to maintain a stable dye-to-cell ratio.

There is discrepancy in measurement due to tissue source. DNA measurements can be made from fresh cells, frozen cells or cells that have been paraffin-embedded for preservation. (Recall that use of paraffin-embedded tissue has made it possible to analyze flow cytometry data with long-term follow-up data.) DNA flow cytometric measures are comparable between fresh cells and frozen cells, but these are not necessarily comparable with the measures taken from paraffin-embedded cells (Frierson, 1988; Hitchcock and Ensley, 1993; McIntire, Goldey, Benson and Braylan, 1987; Zalupski et al., 1993; Pelstring, Hurtubise and Swerdlow, 1990). Other authors have found that the measurements from fresh or frozen cells are comparable to those from paraffin-embedded tissue (Alanen et al 1989; Weaver et al., 1990). In addition, Kenyon et al. (1994) found DNA analysis to be more precise when analyzing a sample that had been sorted to be rich in tumor cells.

The processing of paraffin-embedded tumor cells has many steps and choices of procedure within each step. Even the thickness of the slice of tissue can affect the amount of debris present in the cell suspension (Hitchcock and Ensley, 1993). Pres-

ence of a false aneuploid peak can be the result of choice of fixative (Carr and Abaza, 1993; Herbert et al., 1989; Pelstring, Hurtubise and Swerdlow, 1990; Shankey et al., 1993). Autolysis can yield poor quality data sets, skewed diploid peaks, false aneuploid peaks, increased debris, artifactual antigen expression and changed chromatic structure which alters the stoichiometry of dye binding to DNA (Alanen, Joensuu and Klemi, 1989; Hitchcock and Ensley, 1993; Joensuu, Alanen, Klemi and Aine, 1990; Rabinovitch, 1993). False aneuploidy has been observed in fixed, paraffin-embedded normal tissue (Rabinovitch, 1993; Joensuu et al., 1990).

Different methods can be used for the isolation of cells into a cellular suspension (disassociation) for the flow process: chemical, mechanical, enzymatic digestion. Usually paraffin-embedded tissue is subject to enzymatic digestion, but here, again, the choice of the enzyme and the protocol used has an effect on the DNA measurements (Chassevent et al., 1984; Hitchcock and Ensley, 1993; Hitchcock and Scott, 1990, 1991). Different enzymes, as well as different strengths of the same enzyme and time exposed to the enzyme, can cause differences in the amount of aggregation and debris present, in the quality of the data set and in the estimate of the S-phase fraction. In addition, choice of enzyme and enzyme process can cause a significant difference in the staining intensity.

The setup of the flow cytometer contributes to the variability of the data. Alignment of the laser beam and the cell suspension is essential. But there are also optical factors, including the sensitivity of the photodetectors, and the properties of the fluorochromes and lasers being used, which affect the quality of the data. There is empirical evidence (Chance et al., 1995) that the error due to measurement and noise in the system (i.e., from the flow cytometer itself) and the error due to process of the cells (preparation and staining) are not independent.

Wheeless et al. (1989) report interlaboratory variation in DNA histogram variables even when the laboratories were using the same model. The study showed that differences in instrumentation, instrument setup and laboratory techniques, as well as inconsistency of sample preparation, different staining protocols and the timing of the analysis led to variation in estimates of DNA histogram parameters. Instrument performance affects the quality of the DNA data as measured by CV's of the histogram peaks (Shankey et al., 1993). Shankey goes on to point out that linearity of the instrument (recording electrical impulses which are proportional to the intensity of the fluorescence) should be maintained and checked on a regular basis. With many instruments, nonlinearity exists in the lower channels (Rabinovitch, 1993; Shankey et al., 1993). Rabinovitch points out that this can usually be handled by calibrating the instrument or in the software analysis of the histogram.

Reporting of results plays a crucial role in the determination of the prognostic value of DNA cytometric measurements in cancer. Authors need to report details of sample collection and information indicating that the sample is representative, as well as whether a control sample was used; sample handling and preparation including fixation, disassociation technique, stain used and staining protocol; instrumentation used; mathematical model(s) used for histogram analysis, including the methods used for debris and aggregation compensation as well as the model used for assessing quality of data set, estimation of S-phase and detection of aneuploidy. Additionally, information about the DNA measurements needs to be reported, including quantification of histogram quality, the proportion of cells within the peaks and the proportion of the histogram which is due to debris and/or aggregate cells. Currently, values of S-phase estimates vary considerably between laboratories (due to lack of standardized techniques) and between models, hence specific cut points for determination of "high S-phase" and "low S-phase" are meaningless.

In 1984 Hiddemann and co-workers (Hiddeman et al., 1984 a and b) issued an article "Convention on Nomenclature for DNA cytometry" because "terminology used to describe the results of flow cytometry studies is often confusing and not universally applied" ( Shankey et al., 1993). The guidelines suggested in the 1984 article are often not used. Specifically the terminology used with flow cytometry studies should make it clear that the measurements are not direct measurements of chromosomal content. Definition of the S-phase needs to be clearly stated as to whether it is tumor specific, total S-phase, an average of these or S-phase of aneuploid line. In summary, results need to be reported in a way that facilitates comparison between laboratories and between studies.

## 5. CONCLUSION

This paper presents statistical and quality control problems in the analysis of flow cytometric data. The issues are presented in the context of measuring DNA content in tumor cells. Whether flow cytometric measurements will prove to be a valuable tool in clinical prognosis remains to be seen and is probably dependent on type of disease. In order

to determine the value of DNA histogram analysis, reproducibility of results within and between laboratories is critical. The medical literature should also reflect that flow cytometric measures are estimates and as such estimated measurement (and process) error should be cited with results. Statistically sound estimators of the overlapping distributions that make up the DNA histogram and estimation of the variance of these estimators would be a tremendous step forward.

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