

Cytometry of the Cell Cycle: Cycling Through History

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PREHISTORY: THE PAINS OF THE PRE-FLOW CYTOMETRY ERA

For young investigators who presently enter their scientific pursuit focused on cell cycle and have flow cytometry (FCM) at their disposal, it may be difficult to visualize the hardship of experimental procedures during the precytometry era. Autoradiography (1) was then the predominant method for cell cycle studies. It was a cumbersome and time-consuming methodology. The radioisotope-labeled cells deposited on microscope slides had to be fixed and covered with photographic emulsion in nearly total darkness. This was particularly tricky when using the “stripping film” approach, and required the preparer to either be on a carrot diet or to consume large quantities of vitamin A to enhance his or her night vision. After careful air-drying, the autoradiographs had to be left in light-proof boxes for several days’ and sometimes weeks’ exposure. Then, again in the dark, the autoradiographs had to be processed through developer, rinse, and fixer, followed by drying. Subsequently, the cells had to be counterstained through the emulsion (which also was tricky, because the emulsion had a tendency to detach, ruining the specimen) and mounted under a coverslip. Quantitative analysis of autoradiographs was painful as well. One had to identify labeled cells located below the silver grains of emulsion by microscopy, and score by eye the labeling index (LI) by counting hundreds of cells per each sample. Sometimes it was necessary to count individual silver grains, to estimate the intensity of cell labeling. Such analysis could take a long time, leaving the investigator with painful eyes and phantom images of the silver grains residing in his or her retina for hours. Attempts to develop semiautomatic or automatic screening of autoradiographs based on backward light scattering by silver grains of emulsion were generally unsuccessful (1).

Despite the hardship, important discoveries were made, and numerous autoradiographic techniques, designed to assess the cell cycle and kinetics of cell proliferation, were developed. In fact, the evidence that DNA replication is discontinuous during the cycle, occurring within the distinct time interval during the interphase, was obtained by autoradiography (2,3). It was observed that the radioisotope (³²P or ³H)-labeled DNA precursor thymidine was incorporated into nuclei by a fraction of the interphase cells only, leaving many cells with unlabeled nuclei. This finding provided the foundation for subdivision of the cell cycle into four major phases: pre-

DNA synthetic interphase or postmitotic gap (G₁), DNA synthesis phase (S), postsynthetic interphase or premitotic gap (G₂), and mitosis (M). Perhaps the most elegant technique to measure kinetics of cell progression through the cycle was based on pulse-labeling cells with ³H-thymidine, followed by analysis of the percentage of labeled mitotic cells (3,4). Analysis of the kinetics of progression of the cohort of cells labeled during the short pulse in S phase through the narrow time-window of the M phase provided an accurate estimate of the duration of each phase of the cycle and of the whole cell cycle (T_c) (4). In vitro and in vivo applications of ³H-thymidine autoradiography yielded a wealth of information about cell cycle and the kinetics of cell proliferation of several normal and cancer cell models (4–6).

Microspectrophotometry and microfluorometry were also applied in studies of the cell cycle, as techniques complementary to autoradiography. They were used to measure the content of DNA, RNA, and protein in individual cells. However, only few laboratories could afford such instruments, which were then generally homemade. Their development and maintenance required a significant investment and close collaboration of biologists with optical and mechanic engineers. During 1967–1968, one of us (ZD) had an opportunity to use these instruments at the Institute for Medical Cell Research and Genetics at the Karolinska Institute in Stockholm. Directed by Torbjorn Caspersson, the “grandfather” of cytometry (7), this laboratory had the most advanced microspectrophotometers and microfluorometers at the time, and was the Mecca for scientists from all over the world who were seeking a possibility to quantify DNA, RNA, or total protein in individual cells. Reservations to use the microspectrophotometer had to be made weeks ahead, as there was a long waiting line of investigators eager to measure cells. After some experience was gained, approximately 40 cells could be measured per hour. This number was then considered to be adequate for statistical analyses in most publications. Needless to say, the cell analysis had to be biased

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Table 1
Mileposts in Development of Methods to Probe Cell Cycle by Flow Cytometry

Cell attribute(s) analyzed	Cell cycle compartments/events identified	Fluorochrome(s) used	Reference
Univariate analysis of cellular DNA content	$G_{0/1}$ vs S vs G_2/M	Feulgen-Auroamine O, acriflavine	10-12
		EB	13, 14
		PI	15, 17, 18
		Mithramycin,	19
		EB + mithramycin	21,22
		Chromomycin A_3	23
		DAPI	24-27
		Hoechst 33342 (supravital)	28,29
		DHAQ5	30
Bivariate analysis of DNA and RNA content	G_0 vs. G_{1A} vs. G_{1B} vs. S vs. G_2/M	Acridine orange	38-40
Bivariate analysis of ds and ss DNA after partial DNA denaturation (degree of chromatin condensation)	G_0 vs G_1 vs S vs G_2 vs M	Hoechst 33342, pyronin Y	41, 42
		Acridine orange	43
Multivariate analysis of DNA, RNA and protein content	G_0 vs G_{1A} vs G_{1B} vs S vs G_2/M	Hoechst 33342, pyronin Y, FITC	42
BrdU incorporation	S; DNA replication; cell cycle kinetics	Hoechst 33358 quenching	44-47
		AO quenching	48,49
		BrdU immunofluorescence	50,51
		DNA photolysis (SBIP)	52
"Proliferation-associated" proteins	G_0 vs cycling cells	Ki-67 Ab	56
		PCNA Ab	57-59
		Heterochromatin, p105 Ab	60,61
Cyclins	Up to seven "subcompartments" of the cell cycle	Cyclin B1 Ab	64-68
		Cyclin A Ab	
		Cyclin E Ab	
		Cyclins D1, D2, D3 Abs	
pRB phosphorylation	Early postmitotic and G_0	Hypophosphorylated pRB Ab	75
Histone H3 phosphorylation	G_2 vs M	Histone H3 phosphorylated Ab	67,69
DNA damage	ds DNA breaks vs cell cycle phase	Histone H2AX phosphorylated Ab	76

by prior visual selection of the subsequently measured cell.

BEGINNINGS OF FLOW CYTOMETRY: UNIVARIATE CELLULAR DNA CONTENT ANALYSIS

As one can imagine, the possibility of rapid and accurate measurements of cell constituents offered by FCM (8-10), when compared with the pains of pre-FCM era, made the cell cycle investigators extremely joyous. The speed of cell analysis by FCM was four to five *orders of magnitude* greater than slide-based microspectrophotometry. Thousands of cells per sample could be measured instead of only a few cells. The cell selection was unbiased and data analysis was rapid and semiautomatic. Instead of waiting weeks for results of the experiment, e.g., when applying autoradiography, FCM yielded the data almost instantaneously. Given the above, most researchers studying the cell cycle instantly become devotees of FCM. Research progress in cell biology, particularly in the cell cycle field, was dramatically accelerated. The Cell Kinetics Society, which later merged with the Cell Cycle Society to form the Cell Proliferation Society, has become the forum for FCM; most of their members were also members of the Society for Analytical Cytology (SAC; the precursor of ISAC).

Table 1 lists mileposts in the development of FCM methods to probe the cell cycle. Because cellular DNA content alone yields information on cell position in $G_{0/1}$ versus S versus G_2/M , a variety of methods were initially developed to measure this cell attribute (Fig. 1). Historical progress in developing the DNA content using FCM methods was associated with the introduction of new fluorochromes and modifications of the cell staining procedures, as outlined below.

Feulgen-DNA

In early attempts, the Feulgen fluorescent method, utilizing either auramine O (11), or the improved procedure, employing acriflavine (12), was introduced to FCM. As cumbersome and time consuming as the Feulgen procedure was in actual practice, it proved to be of significant value for validating the early applications of FCM. An article appearing in *Science* (10) in 1969 showed a good quality DNA histogram of auramine O Feulgen-stained CHO cells. The coefficient of variation (CV) of the mean fluorescence of G_1 cells reported by these authors (6.4%) was acceptable for that period, and the fluorescence intensity G_2+M to G_1 peak ratio was 1.97, close to the expected value of 2.00. Cell volume distributions were also measured and were different from those for the Feul-

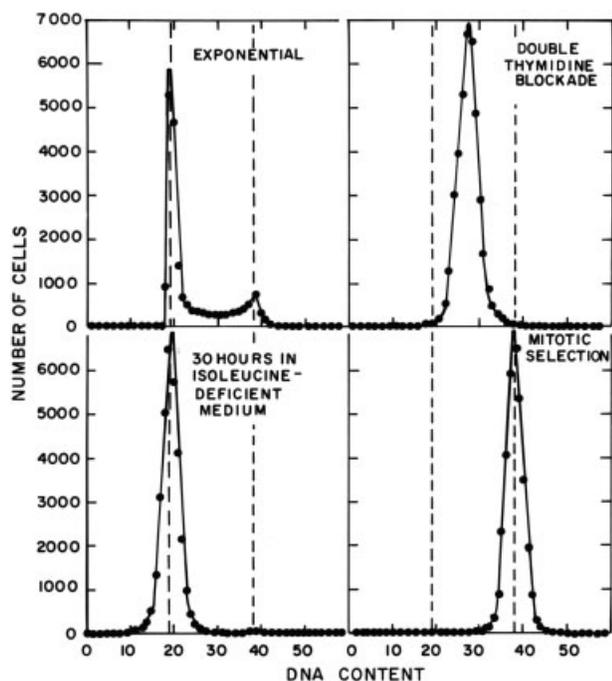


FIG. 1. Composite of DNA content histogram obtained from CHO cell populations in the exponential growth phase, or synchronized in various phases of the cell cycle, as shown. Cells were stained by the acriflavine-Feulgen procedure (11).

gen-DNA content. These data countered the early criticisms of the technique, which implied that the procedure was actually measuring the nuclear or the cell volume.

To further substantiate credibility of the flow cytometric technique, cells were synchronized in the various phases of the cell cycle and analyzed by flow microfluorometry (FMF), as it was often called at the time. The composite in Figure 1 shows histograms of acriflavine-Feulgen-stained CHO cells in exponential growth, synchronized in the early S phase by double thymidine block, arrested in G₁ phase by isoleucine deprivation, or in mitosis by mitotic shake off. This figure was used at the introduction of every presentation by one of us (HAC) to provide validation of the techniques. Still, some skeptics in the audience often expressed doubts whether the cell cycle was indeed being assayed by FCM. However, convinced that the method was reliable and accurate, we used the acriflavine-Feulgen procedure for analysis of cells treated with a variety of chemotherapeutic agents supplied by the National Cancer Institute (NCI). The first paper demonstrating this approach appeared in 1972 (12). Independently, in Europe, Göhde and Dittrich (13) also used FCM to analyze cytostatic affects of antitumor drugs.

Ethidium Bromide (EB) and Propidium Iodide (PI)

Several improved DNA cell-staining techniques were developed in the early 1970s that led to methods for cell cycle analysis that remain in widespread use today. These

methods used the phenanthridine fluorochromes EB and PI. Both dyes intercalate into ds DNA and RNA with a fluorescence enhancement 20–30 times more than the unbound dye. EB was used by Dittrich et al. (14) in combination with fluorescein isothiocyanate (FITC) to stain both DNA and protein, respectively, in cells following enzymatic removal of RNA during the staining reaction. Subsequently, this technique was modified by the substitution of PI for EB (15). This was a novel cytological use of PI and, since the dye was not yet commercially available, a sample was obtained gratis from Dr. B. Hudson and colleagues, who had synthesized PI for use in procedures for the isolation of closed circular DNA (16). Both EB and PI are cell-impermeant and ethanol fixation was required for the above procedures. However, Krishan (17) later used PI in a hypotonic citrate solution, which essentially disrupted the cell membrane so that fixation and RNase treatment of the cell samples was not required. Data obtained from analysis of cell populations obtained from solid tumors grown subcutaneously in mice and stained using this procedure is shown in Figure 2. The hypotonic method was simple and rapid; these were the virtues that contributed to its popularity. The protease/detergent method subsequently developed by Vindeløv et al. (18) provided consistently high resolution of DNA content measurement (lower CV) and was more often used for analysis of clinical samples.

Mithramycin

Another rapid cell staining procedure resulted from somewhat incident findings of one of us (HAC), obtained while studying the effects of the fluorescent antibiotic mithramycin (MI) on cell cycle progression. Subsequent investigations demonstrated MI-specificity for DNA in single cells, and a procedure was developed using MI in a simple solution containing phosphate buffered saline (PBS), magnesium chloride, and 25% ethanol (19). Cells were thereby permeabilized and stained in one step, and cell cycle analysis could be performed in a total of 20 min. The MI procedure received substantial application in the 1970s, but the requirement for a 5.0-W argon laser to provide adequate 457-nm excitation of the fluorochrome has limited its usage today. The DNA content histogram in Figure 3, obtained using the MI staining technique, shows the Dean and Jett (20) computer-fit modeling used to obtain the percentage of cells on S phase from the histogram. For comparison, Figure 3 also shows the percentage of S phase cells obtained by ³H thymidine labeling and autoradiography. A combination of MI and EB as DNA stain, introduced by Barlogie et al. (21), was reported to provide better DNA content resolution than each of these dyes alone (22). Chromomycin A₃, an antibiotic with a chemical structure similar to MI, was used by Jensen (23) for cell cycle analysis of human gynecological samples.

4'-6-Diamidino-2-Phenylindole (DAPI)

The UV-excited fluorochrome DAPI was introduced to FCM by Stöhr et al. (24) and Göhde et al. (25). Among several DNA fluorochromes compared, binding of DAPI to

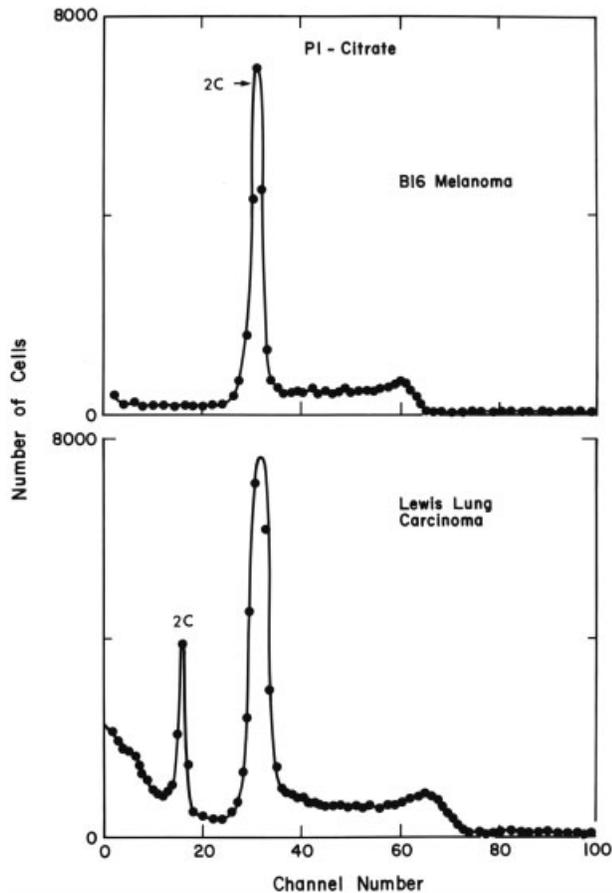


FIG. 2. DNA content histograms obtained from B16 melanoma and Lewis lung carcinoma cell populations stained with PI in hypotonic citrate (17).

DNA was shown to be the least affected by differences in composition and structure of nuclear chromatin (26). DAPI, therefore, appears to be the preferred fluorochrome for quantitative analysis of cellular DNA content in different cell types, regardless of structure (degree of condensation) of nuclear chromatin. In concordance with this observation (26), the highest resolution of DNA content histograms (the lowest CV values of the mean DNA content of uniform cell populations) was reported for the cells, including spermatozoa, stained with DAPI (CV = 0.5–1.0%) (27).

DNA Staining in Live Cells

Early studies utilized impermeant fluorochromes that required cell fixation—a step incompatible with cell viability. Hilwig (28) introduced Hoechst 33258 and Hoechst 33342 as supravital DNA fluorochromes and Arndt-Jovin and Jovin (29) used them in FCM and to sort live cells on the basis of cellular DNA content. The Hoechst dyes require excitation in the UV light range. Recently, Smith et al. (30) have shown that another fluorochrome, DRAQ5, also is able to stain live cells. This far-red fluorescing dye can be excited in the 488–647 nm range. However,

DRAQ5 appears to be fairly toxic, so the long-term viability of the stained cells is limited. Despite that limitation, DRAQ5 is expected to find a host of applications, since, unlike Hoechst dyes, it does not require UV excitation. The success of permeant DNA fluorochromes that can be used for live cells depends on either nonfluorescent binding to RNA and other polyanions, or not binding. Hoechst 33342 binds to RNA, but does not fluoresce (Jacobberger, unpublished).

DNA Content FCM of Paraffin-Embedded Tissues

Knowing how difficult it is to isolate intact cells or cell nuclei from fresh tissue, one has to be an extreme optimist to attempt to isolate cells or nuclei from paraffin-embedded tissues. Certainly such an optimist was Hedley et al. (31), who developed the methodology that allows one to subject cell nuclei isolated from paraffin blocks to FCM analysis. This technique opened unlimited possibilities to analyze archival specimens stored in pathology departments for retrospective studies. There are some limitations to this methodology, primarily related to variability in fixation procedure (e.g., duration, temperature, formaldehyde strength, specimen size, and tissue type), which in turn affect DNA stainability after nuclei isolation. Nevertheless, the methodology became widely used and helped to attract legions of pathologists, who used FCM primarily to explore whether the frequency of S phase cells (indicative of a higher fraction of cycling cells) or the presence of more than one stem line in cancers (DNA-aneuploidy) may be prognostic indicators.

Early Data Presentation and Analysis

Prior to the development of DNA histogram deconvolution algorithms to analyze the FCM data, the investiga-

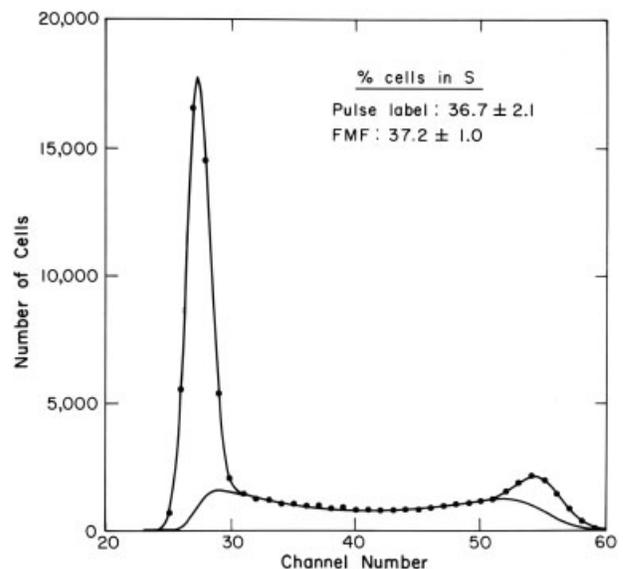


FIG. 3. DNA histograms of CHO cells stained with MI (19). The percentage of cells in S phase as obtained by the computer fit model of Dean and Jett (20) from FMF, was compared to the percentage of cells obtained by pulse-labeling with tritiated thymidine and autoradiography.

tors had to improvise and develop their own approaches. The most common was integration of the cell count by computer upon setting the gates for G_1 versus S versus G_2/M cell populations "by eye." Accuracy, objectivity, and reproducibility of such an approach were debatable, and reviewers of these papers had an open field to criticize the analysis. Needless to say, often the coauthors had different opinions and vehemently argued between themselves as to where to set the gates, particularly when cell populations were synchronized and the histogram did not show the distinct G_1 and G_2/M peaks. Another approach was to copy outlines of the histogram from the image on an oscilloscope onto uniform thickness filter paper. The peaks and ridges of the histogram related to G_1 versus S versus G_2/M cells were then cut off and weighted. Their weight represented the integrated number of cells in respective phases of the cell cycle. Obviously, sharp scissors and accuracy of the balance were as essential for precision of DNA measurement as good histochemistry and a good flow cytometer. One of us (JWJ, Ph.D. thesis) used this paper cutting technique to measure the cell cycle stages of malaria parasites. This work was rejected from a parasitology journal, with a reviewer stating that the paper cutting method was "unsophisticated." When the ModFit (Verity Software House, Topsham, ME) flexible modeling software was introduced, the same data were analyzed in a "sophisticated" manner and was subsequently published (32). The results obtained by the two methods were highly correlated and provided identical biological information.

Reproduction of the DNA histogram image that appeared on the oscilloscope screen of the pulse-height analyzers connected to the early FCM system was time consuming and expensive. A hooded Polaroid camera was used to take a photograph of the histogram and a protractor was then used to expand and scale the histograms onto graph paper. This figure was then provided to a draftsman who drew and labeled the axes and reproduced the histogram with printer's ink. Photographs of the figure provided the 8×10 inch glossy required for the publication, and the 35-mm slide for the presentation. The process sometimes took one to two weeks.

Histogram Deconvolution Software

The progress in computer capability and mathematical proficiency of some of our colleagues who were interested in the cell cycle led to the development of algorithms to deconvolute DNA histograms. For mammalian cells, Gaussian distributions were used to model the $G_{0/1}$ and G_2+M fluorescence distributions and either a polynomial (20) or a trapezoid (33) function was used to fit the S phase. A third, less popular fitting routine that works well for synchronized cells, uses multiple Gaussian distributions to fit the S phase (34). In all programs used today, the S phase functions are tailed with Gaussian distributions. This improves the statistical accuracy of S phase fraction estimates. Two commercial computer programs in wide use are ModFit LT written by Bruce Bagwell (Verity Software House, Topsham, ME) (33) and Multi-

Cycle written by Peter Rabinovitch (University of Washington, Seattle, WA) (35). These programs now have sophisticated functions to estimate the degree of overlapping distributions that can be attributed to debris, aggregates, and nuclei that have been cut (35). These rule-based analytical approaches provide accurate measurements from data that can be distressfully obscured by unwanted events, which are often present, e.g., in samples of nuclei extracted from paraffin-embedded blocks (31) or freshly dissociated tissue.

DNA Content in Clinical Samples

For a time, DNA analysis was done extensively on clinical tumor samples, with many results strongly suggesting that the presence of DNA-aneuploid stem lines and high S phase fractions were predictive of poor outcome for many tumors. However, the seemingly simple technique of dissociating and staining cells was difficult for many laboratories to master and many studies were reported that were erroneously evaluated. The result of this was a large conflict in estimating the clinical value and utility of single parameter DNA analysis. This resulted in an issue of *Cytometry* (1993, Volume 14, Issue 5) that was devoted to a consensus conference of interested experts seeking to either discover the truth or at least find consensus. Although the consensus was that DNA content measurements added informative prognostic information for several tumors, a later report concluded that DNA content measurements for breast and colon cancers should not be routinely employed, noting that many studies were explorative in nature, that multiple S phase cutoffs were used for high and low risk groups, S phase and ploidy were correlated, and that there was a lack of prospective studies (36). More recently, Bagwell et al. (37) showed both independence of S phase and ploidy as well as the improved prognostic significance of DNA content measurements on breast cancers. The approach here was rule-based analysis with 10 adjustments to standard procedures that were developed on one large retrospective study ($N = 935$), then applied to two others (210 and 220 cases) with equivalent results.

ACRIDINE ORANGE: THE "MAGIC" FLUOROCHROME IN ANALYSIS OF THE CELL CYCLE

While univariate DNA content analysis made it possible to distinguish $G_{0/1}$ versus S versus G_2/M cells, it could not discriminate between cells having the same DNA content such as quiescent (G_0) cells versus G_1 or G_2 versus M cells. Frequently, however, identification of these cells is desirable. The first cytometric methods that were able to identify these cells utilized acridine orange (AO), the metachromatic fluorochrome that under the right conditions of equilibrium staining can differentially stain ss versus ds nucleic acids (38-40). Namely, AO binds to ds nucleic acid by intercalation and this binding manifests in green fluorescence (~ 530 nm). Its interactions with ss nucleic acid, on the other hand, involve condensation of the product, which then fluoresces red (~ 640 nm). There-

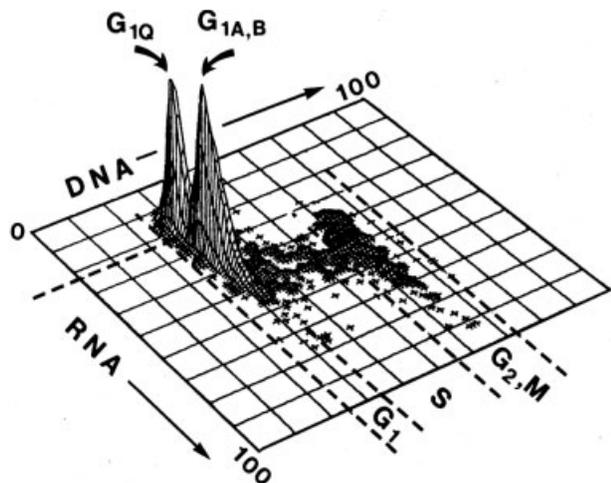


FIG. 4. Discrimination of G_{1Q} , G_{1A} , G_{1B} , S, and G_2/M subcompartments of the cell cycle of mitogenically stimulated lymphocytes, based on differential staining of DNA and RNA with AO (38-40). The increased RNA content of cycling G_1 cells compared to quiescent G_0 (G_{1Q}) cells is quite evident. It is also apparent that G_1 cells enter S phase only when the threshold amount of RNA has been accumulated; this threshold distinguishes G_{1A} from G_{1B} cells (see [39,40] for details).

fore, after selective denaturation of RNA, AO can differentially stain DNA (green) versus RNA (red) (38-40). Since over 95% of total cellular RNA is rRNA, and quiescent (G_0) cells have several-fold fewer ribosomes compared to their cycling G_1 counterparts, following differential staining of DNA versus RNA with AO, G_0 cells were identified as the cells with minimal RNA content (Fig. 4). It was also possible to distinguish early G_1 cells (G_{1A}) from the late G_1 (G_{1B}) cells, based on a characteristic threshold in RNA content that character-

ized the cells entering S phase (39). Interestingly, in some cell systems, the cells that were quiescent (non-cycling) had an S or a G_2/M DNA content; such cells also had minimal RNA content and thus they could be distinguished from cycling S and G_2/M cells (40). The same cell cycle compartments were subsequently identified using Hoechst 33342 and pyronin Y as DNA and RNA fluorochromes, respectively (41,42).

The first cytometric method that allowed one to distinguish G_2 from M cells also utilized AO (43). However, in this technique, RNA was removed by prior incubation of cells with RNase and DNA was partially denatured by heat or acid. When such cells were stained with AO, the nondenatured ds DNA fragments bound the fluorochrome by intercalation, fluorescing green, while the denatured ss fragments had red fluorescence (43). It had been noticed that DNA in condensed chromatin of M cells is much more predisposed to undergo denaturation when heated or acid-treated, compared to DNA of G_1 , S, or G_2 cells. Furthermore, DNA in condensed chromatin of G_0 cells was more prone to undergo denaturation compared to DNA of G_1 cells. This method, thus, allowed one to distinguish all five cell cycle compartments, G_0 , G_1 , S, G_2 , and M (Fig. 5).

Thus, the magic fluorochrome AO that was able to probe two relatively nonspecific cell attributes such as cellular RNA content and degree of DNA denaturation, each combined with measurement of DNA content, has become a convenient tool used in studies of the cell cycle. The cell cycle compartments identified by this dye reflected changes in number of ribosomes or degree of chromatin condensation as the cells entered the cycle from G_0 and progressed through G_1 , S, G_2 , and M.

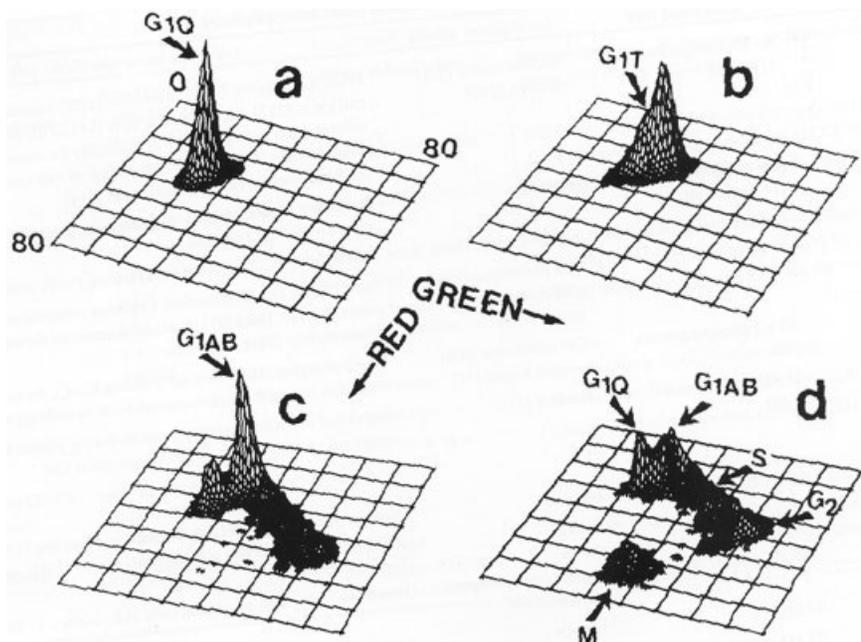
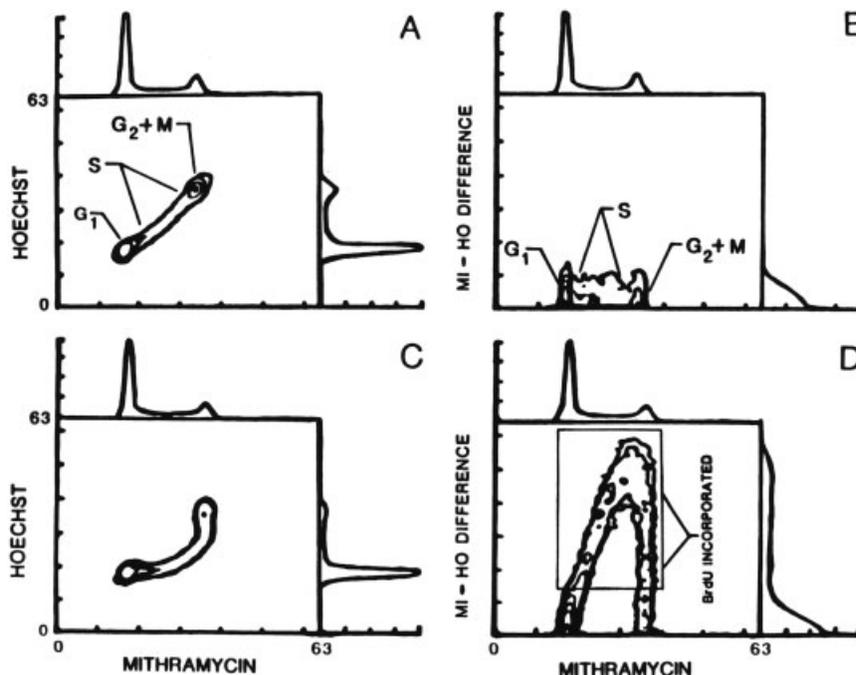


FIG. 5. DNA acid-induced denaturation pattern of unstimulated and mitogenically-stimulated lymphocytes. Unstimulated lymphocytes form a uniform G_{1Q} population (a). Early during stimulation (18 h) cells undergoing transition to proliferation (G_{1T}) are characterized by increased green (ds DNA) and decreased red (ss DNA) fluorescence (b). Additionally, subpopulations of S, G_2 , and M cells can be distinguished on the third day of stimulation (c,d). The culture shown in (d) was treated with colcemid for 8 h to arrest cells in M; note the increase in frequency of M cells and the decrease in G_{1AB} cells. The changes in in situ DNA propensity to denaturation reflect changes in chromatin condensation (40,43).

FIG. 6. DNA content (Hoechst [HO] and MI) and the MI minus HO signal difference frequency distribution histograms and the corresponding bivariate contours for untreated (A,B) and for CHO cells treated with 30 μ M BrdUrd for 30 min (C,D). The quenching of HO in S phase cells can be noted in (C) and the significant MI minus HO signal differences in S phase cells is readily apparent in (D). (See [47] for details).



FLOW CYTOMETRY OF DNA REPLICATION-BrdU INCORPORATION

As has been already pointed out, advantages of using flow cytometry to detect DNA replication can only be fully appreciated by the investigators who used ³H-thymidine autoradiography for the same purpose. Three methodological approaches characterized progress in this area. The first approach was based on the discovery that fluorescence of Hoechst 33358 bound to DNA is reduced ("quenched") in cells that incorporated the thymidine analogue 5'-BrdU (44). The BrdU-labeled cells, therefore, could be identified based on the deficit in Hoechst 33358 stainability, compared with their DNA content. Differential DNA staining with Hoechst 33358, combined with the dye whose fluorescence is unaffected by the incorporated BrdU, such as EB, made it possible to identify BrdU-incorporating cells concurrently with analysis of the cell cycle distribution of the measured cell population (45). Different strategies were developed, using pulse- and cumulative BrdU-labeling to estimate many parameters of the cell cycle kinetics (46). One of the distinct advantages of this method for kinetic studies was that multiple division cycles could be followed. The sensitivity of this method was improved by using MI instead of EB, and by using the strategy of subtraction of Hoechst 33358-fluorescence signals from MI-fluorescence signals (differential fluorescence) as a separate parameter (47) (Fig. 6).

Because (like Hoechst 33358) fluorescence of AO bound to DNA is also quenched by BrdU (48) by combining BrdU incorporation with identification of M cells based on DNA denaturation assay (43), it was possible to distinguish the BrdU-labeled from -unlabeled M cells (49). This approach opened a possibility of the use of FCM for

the fraction of labeled mitosis (FLM) assay to analyze cell cycle kinetics (3,4).

The second approach to detect DNA replication by flow cytometry was based on immunocytochemical detection of BrdU (50). In this method, following partial DNA denaturation by strong acid (2 M HCl) or heat, the incorporated BrdU becomes accessible to BrdU Ab. The latter is most frequently, directly or indirectly, labeled with fluorescein. When the nondenatured DNA sections are counterstained with a red fluorescing fluorochrome, such as PI, the bivariate distributions reveal both cellular DNA content and the presence of incorporated BrdU (51).

Immunocytochemical detection of the incorporated BrdU requires the harsh step of DNA denaturation. This treatment often ruins cell morphology, and by damaging many epitopes, precludes immunocytochemical detection of other markers. The third approach developed for BrdU detection omits the DNA denaturation step and is therefore compatible with a concurrent use of other immunocytochemical probes. This is the strand break induction by photolysis (SBIP) method (52), which relies on illumination of the cells with UV light to selectively induce photolysis (DNA breaks) at DNA sections that contain the incorporated BrdU. DNA strand breaks generated by photolysis are then labeled with the fluorochrome-tagged deoxynucleotide by the terminal deoxynucleotidyl transferase, similarly as in the TUNEL assay, to detect DNA breaks in apoptotic cells (Fig. 7) (53). Kits are now commercially available (ABSOLUTE-SBIP; e.g., Molecular Probes, Eugene, OR) that rely on this methodology.

Because of a high signal to noise ratio imparted by immunofluorescence, and because a blue, rather than UV laser, is required, the immunocytochemical detection of

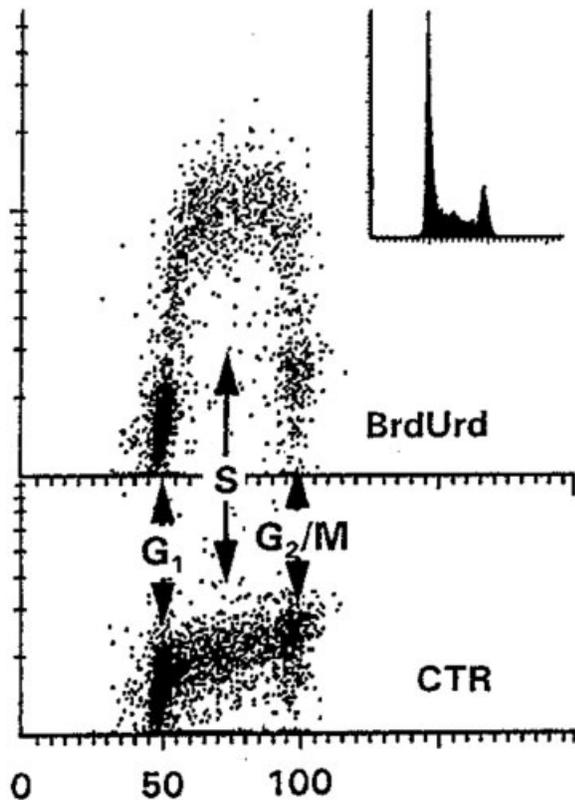


Fig. 7. Detection of BrdU incorporation by the SBIP method (52). The cells were incubated with BrdU for 60 min, their DNA was photolyzed by exposure to UV light (incorporated BrdU sensitizes DNA to UV light) and the photolysis-generated DNA strand breaks were labeled with fluoresceinated deoxynucleotide using exogenous terminal deoxynucleotidyl transferase; DNA was counterstained with PI. Bottom (CTR) scatterplot shows cells that were not incubated with BrdU.

BrdU incorporation has become the predominant methodology to assess DNA replication, and in particular, to study kinetics of cell cycle progression. Several mathematical models have been developed to follow the cohort of cells pulse-labeled with BrdU through different cell cycle phases to obtain the kinetic parameters (54,55). Of special value in the clinic is the method developed by Begg et al. (54) to measure duration of S phase and potential doubling time of tumor cells, from a single sample collected at a certain point in time after *in vivo* administration of BrdU.

PROLIFERATION-ASSOCIATED PROTEINS

As mentioned, rather nonspecific cell attributes such as RNA content and degree of chromatin condensation, were found to be useful probes to distinguish quiescent from cycling cells and identify several compartments of the cell cycle. The subsequent steps in probe development were focused on immunocytochemical markers, expected to offer higher specificity toward particular components of the cell cycle progression machinery. Initially, prior to discovery of cyclins and CDKs, antibodies to so-called "proliferation-associated antigens" have been produced and tested on different cell systems, primarily to assess

their utility as diagnostic and prognostic markers of tumor cell proliferation. Some of these antibodies were isolated from sera of patients with autoimmune diseases such as systemic lupus erythematosus, others were raised against whole cell nuclei or nuclear extracts from proliferating or quiescent cells. Initially, little was known about intracellular antigens reactive with these antibodies and their function during the cell cycle. The most widely recognized became Ki-67, the antibody that is now being considered to be a reliable marker distinguishing cycling from noncycling cells and that is often used to measure tumor cells' growth fraction (56). Although the function of the protein reactive with this Ab (pKi-67) is still an enigma, its clinical value in identifying tumor cell proliferation potential is generally accepted.

Another proliferation-associated protein that found wide application as a marker of cell proliferation is proliferating cell nuclear antigen (PCNA) (57). PCNA was later characterized as a multifunctional protein that, among many functions, is a part of the DNA polymerase holoenzyme, interacts with CDKs, cyclins, and CDK inhibitor p21 to form ternary complexes, and participates in nucleotide excision DNA repair (58). During S phase, PCNA is chromatin-bound, remaining in the nucleus following treatment with detergents. Cell lysis by detergent, therefore, followed by their fixation and immunocytochemical detection of PCNA concurrent with differential staining of DNA results in bivariate distributions that allow one to identify DNA replicating cells and classify cells into G_{0/1}, S, and G₂M, based on DNA content, similar to DNA-BrdU distributions (59).

Still another nuclear antigen whose abundance changes during the cell cycle progression is p105, identified by Bauer et al. (60) and Clevenger et al. (61). This protein appears to be associated with heterochromatin, and is highly expressed in cells having condensed chromatin, such as M and G₀ cells. In fact, p105 antibody has been used in pioneering studies to immunocytochemically identify M cells and score mitotic indices by flow cytometry (61). Similar to Ki-67, the function of p105 is not well understood.

CYTOLOGY OF CYCLINS AND OTHER COMPONENTS OF CELL CYCLE REGULATION

Cyclins, CDKs, inhibitors of cyclin-dependent kinases (CKIs), the retinoblastoma family proteins (Rb), and ubiquitin-mediated proteolysis complexes (APC, SCF) comprise the core components of the molecular machinery that drives the cell through the cell cycle. Some of these proteins are expressed discontinuously during the cycle or are induced (or suppressed) by growth factors, mitogens, differentiating agents, or others (62,63). Their immunocytochemical detection thus provides a very specific marker of cell position in the cycle and of its potential for proliferation.

Immunocytochemical detection of cyclins D, E1, A2, and B1, combined with DNA content measurement, opened a number of new opportunities for probing the cell cycle (Fig. 8) (64-67). Thus, it became possible to

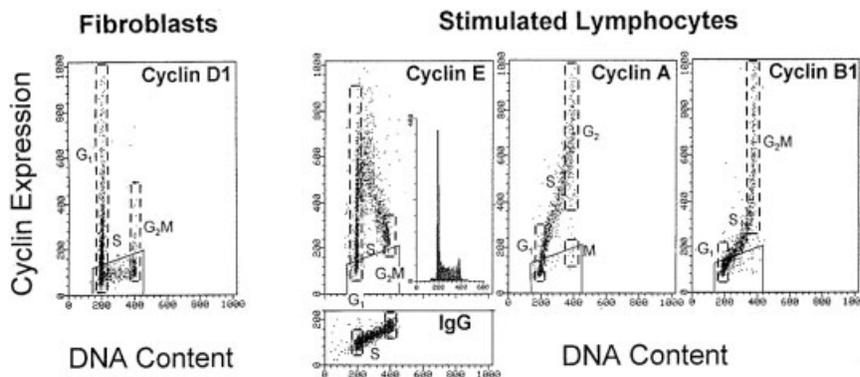


FIG. 8. Characteristic bivariate distributions showing cyclin immunofluorescence (expression of cyclins D1, E, A, and B1) versus cellular DNA content of exponentially growing normal fibroblasts and lymphocytes (67).

distinguish G_1 cells of higher DNA ploidy from G_2 cells of lower DNA ploidy having the same DNA content. It also became possible to discriminate between G_2 and M cells, the latter were identified within a well defined time-window between prometaphase and cytokinesis. The lack of cyclin D and cyclin E expression among the cells with a G_1 DNA content marked the noncycling G_0 cells with hypophosphorylated Rb. Altogether, it became possible to identify cells in six to eight cell cycle compartments differing in their degree of progression through the cycle (67).

The most important role for multiparameter flow cytometry in analysis of cyclins was the possibility it offered to identify cells expressing cyclins in an “unscheduled” mode, namely G_1 cells expressing G_2 cyclins (cyclins A and/or B1), and G_2 cells expressing G_1 cyclins (cyclins E and/or D), or the lack of expression of some cyclins (65,66). Such unscheduled expression is characteristic of some tumor cell types but may also be induced in normal cells, e.g., by their arrest in the cell cycle, causing unbalanced growth (67). While the prognostic value of unscheduled expression, or lack of expression, of some cyclins remains to be elucidated, it has to be stressed that multiparameter cytometry is *the only approach* that allows one to detect and quantify it. The alternative method, Western immunoblotting, is unable to correlate cyclin expression with the cell cycle phase of individual cells, or to eliminate the contribution of stromal or infiltrating normal cells in the case of tumor samples.

Recent work in this area suggests that multiparametric cytometry can provide a means to parse cell cycle regulation in fine and somewhat unexpected detail. In this paragraph the findings from the laboratory of one of us (JWJ), largely unpublished, are outlined. Namely, we have been able to quantify the fraction of cells in G_1 , S, G_2 , prophase, prometaphase, metaphase, and late mitosis, and to identify two new cell cycle compartments defined by cytometry. This was accomplished using mitotic markers like phospho-histone H3 and MPM-2, combined with cyclin B1 and cyclin A (as reviewed in 68). Histone H3 is phosphorylated at Ser-10 by Aurora B kinase (68), and appears to be the best marker for mitosis (70). MPM-2 is also a phospho-epitope, which is expressed at a high level

on many different proteins in mitosis (71). Expression of this epitope increases later than phosphorylation of Ser-10 of histone H3 (pH3), and the cytometry of MPM-2 and pH3 defines a new cell cycle compartment located past G_2 and prior to a cytometrically defined transition state that corresponds to prophase (Jacobberger and Sramkoski, unpublished data). Prophase cells are characterized by peak or near peak levels of cyclin A and cyclin B1 (72). When measured by cytometry, a distinct cluster with prophase characteristics can be detected. Since the transition from prophase to metaphase is characterized by the activity of a form of APC that degrades cyclin A, prometaphase cells can be detected as cells with lower than peak levels of cyclin A, but peak or near peak levels of cyclin B1. Metaphase cells can be detected as a cluster defined by undetectable cyclin A and peak levels of cyclin B1. Cyclin B1 accumulates until metaphase and is destroyed by APC at the onset of anaphase (73,74). The mitotic stages past metaphase are not clearly resolved by cytometry, with the exception of a population of cells at cytokinesis that are identified by doublet pulse-width discrimination. We have also been able to detect new-born G_1 cells in human tumor cell lines. These are G_1 cells that enter G_1 and still express a fraction of the mitotic intensity, either pH3 or MPM2. Thus, with the use of three epitopes—in this case, all measuring the activity of regulatory enzymes—and DNA content, the cell cycle can be dissected in a more complete manner. One interesting aspect is that the data are distinctly clustered, angled in parametric space, and transition states between clusters are populated. It is possible, therefore, that in the future the cell cycle will be measured, similar to immunophenotyping for hematopoietic cells, with defined boundaries of expression of detectable enzyme activity to define the existence of cells in cell cycle states. A representative analysis, illustrating this concept is shown in Figure 9.

It should be noted, however, that whereas such dissection of the cell cycle applies to normal cells, some tumor cell types may have unscheduled expression or phosphorylation of these proteins, similar to what occurs with the expression of some cyclins (65–67), which may preclude universal applicability of such an approach.

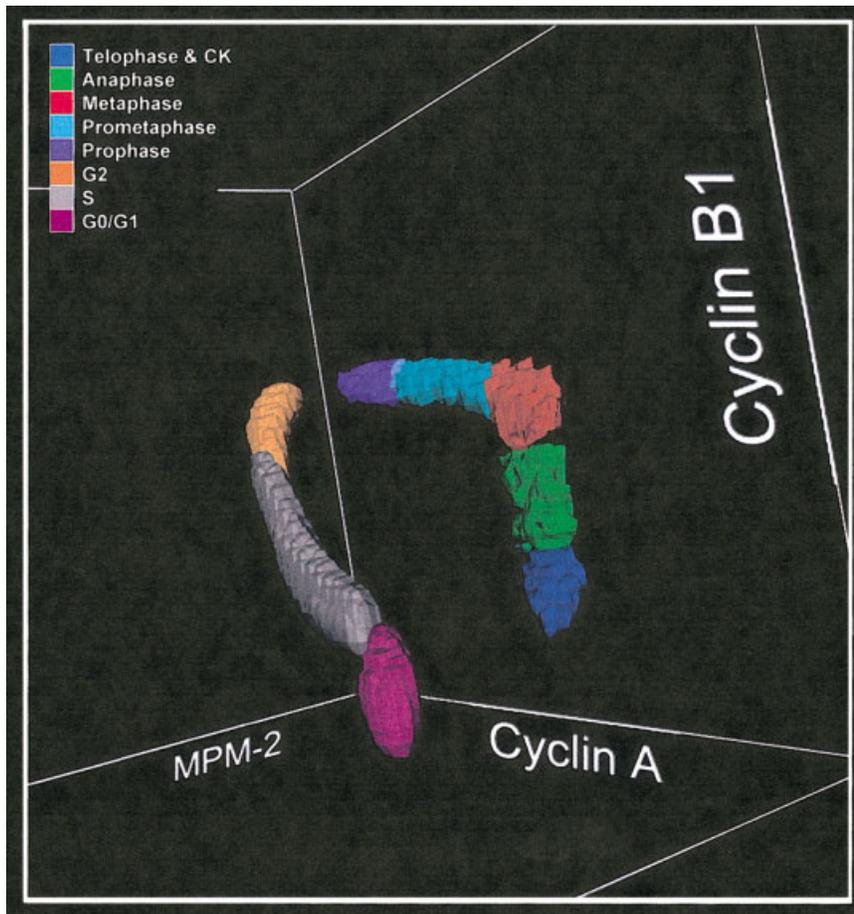


FIG. 9. Four parameters, DNA content (not shown), MPM-2, cyclin A, and cyclin B1, were measured by FCM to distinguish the cell cycle compartments as marked. The G_1 boundary was defined by DNA versus cyclin A. The G_2 boundary was defined by DNA versus cyclin A AND (Boolean AND) DNA versus MPM-2. The mitotic subphases were defined by gating on a tight maximum cluster of MPM-2 expression at a $4C$ DNA content, plotting cyclin A versus cyclin B1, and gating the clusters and transitions between them. The clusters are defined at the 80th percentile of the two-parameter plot from which they were derived, and therefore, the size of the clusters in the plot are not proportional to the frequency of events in each cluster in the entire population of cells. The clusters labeled prometaphase, anaphase, and telophase/CK are enriched for these cell types, but are not as clearly defined as either the prometaphase or metaphase clusters.

FUTURE DIRECTIONS: FUNCTIONAL PROBES

Future applications of cytometry in studies of the cell cycle will expand in many directions. We do already see rapid development of immunocytochemical probes that detect functional state of particular proteins, related to their chemical modification that alter their conformation and activity, such as phosphorylation, acetylation, ubiquitination, cleavage, and others. Probes of this kind have already been used in multiparameter cytometry for cell cycle analysis (68,70,75-77). The ratiometric analysis of the combined probes, one that measures abundance of a particular protein, and another reporting the protein modification, will open further avenues to probe the functional state of the cell related to its cell cycle position. Using the ratiometric approach Juan et al. (75) were able to measure changes in abundance of Rb combined with the degree of its phosphorylation in mitogenically stimulated lymphocytes during their transition from G_0 to G_1 .

In addition to overall expression and chemical modification of a particular protein, its functional status depends on subcellular localization, and therefore is modulated by its translocation. The inducers or suppressors of transcription, for example, have to be transported into the nucleus to affect transcription of the respective genes. Their activity can thus be assayed by monitoring the translocation.

The instrumentation that allows the morphometric cell analysis, such as laser scanning cytometry (LSC), or similar imaging cytometers, has to be employed in such assays. In fact, LSC has already been used to monitor translocation of cyclin B1 (78) and NF- κ B (79) to the nucleus, cyclin E to the nucleolus (80), and the proapoptotic protein Bax into mitochondria (81), all in relation to the cell cycle phase.

The major advantage of multiparameter cytometry stems from its ability to provide multivariate analysis of several cell attributes measured concurrently in the same cells. This feature is unique to the methodology, as it allows one to even probe a possible cause-effect relationship between the measured attributes. Namely, individual cells are notoriously asynchronous in all their functions with respect to each other, within a cell population. Thus, if event A precedes event B, but is not a prerequisite for event B to occur, although a predominant number of cells expressing B will also express A, many cells in the population will be B-positive/A-negative. An exception, of course, would be if event A is transient and rapidly becomes undetectable. However, when event A is a precondition for event B to occur, and is relatively stable, essentially all B-positive cells are also expected to be A-positive. With molecular probes of function/activity of individual proteins now becoming available, multiparameter cytometry

etry, by offering a unique opportunity to investigate the interrelationship between measured events in single cells, will continue to be the essential methodology in cell cycle studies, complementing classical biochemical and molecular biology methods.

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