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Award Number: DAMD17-00-1-0342

TITLE: Multiparametric Evluation of Marker Expression

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REPORT DATE: June 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2001	Annual (1 Jun 00 - 30 May 01)

4. TITLE AND SUBTITLE
Multiparametric Evaluation of Marker Expression

5. FUNDING NUMBERS
DAMD17-00-1-0342

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

During first year of this project, we have carried out multiparametric flow analysis of estrogen receptor (ER) expression in 75 formalin fixed/paraffin embedded human breast tumors. Flow cytometric data was compared with immunohistochemical studies and correlation between ER expression and the histopathological evaluation was undertaken. A manuscript is being prepared for publication.

We have developed a high-resolution flow cytometer for measurement of nuclear volume and DNA content of nuclei. We developed a staining protocol for simultaneous evaluation of nuclear volume and DNA content of nuclei. A manuscript was published. We have analyzed 50 fresh/frozen tumor biopsies for nuclear volume versus DNA content and a manuscript is being prepared for publication.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Flow cytometry, Hormone Receptors, Estrogen, Progesterone, breast	17
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION: Flow cytometry (FCM) is an important method for monitoring of cellular receptor expression in hematological tumors and for the determination of DNA content and proliferation in breast tumors. However, use of FCM for monitoring hormone receptor expression in breast tumors has been limited, mostly due to the difficulty in obtaining single cell suspensions, reduced reactivity of the receptor antigens in formalin fixed tissues and the poor reactivity of the antibodies (primarily selected for immunohistochemical analysis).

These methods will be rapid / highly sensitive and can determine not only the percentage of receptor positive cells in a heterogeneous population but also measure antigen density on the individual cells and subpopulations. We believe flow cytometric analysis of hormone receptors and other cellular markers can be an important quantitative and multiparametric method for diagnostic and prognostic studies in breast cancer.

BODY:

1. Estrogen Receptor Expression and DNA content of Human Breast Tumors: Specific aims of the year 01 were to:

1. Collect breast tumor biopsies and archival tissue blocks from local and outside repositories.
2. Refine methods for analysis of single cells and nuclei obtained by enzymatic digestion of archival breast tumor biopsies for hormone receptor expression.
3. Study the resulting single cell nuclei for marker expression (Estrogen receptor, DNA content and Electronic nuclear volume).

During this year we collected fresh breast tumor biopsies from Jackson Memorial Medical Center, Miami (13), from Suburban Hospital, Bethesda, MD (15) and from the NIH sponsored CHTN tissue network, Birmingham, AL (35).We also collected 388 blocks formalin fixed/paraffin embedded blocks of breast tumors representing a variety of histopathological grades and types of tumors for our project.

Thick (50 micron) and thin (5 micron) sections were cut from seventy breast tumor samples for analysis by immunohistochemistry and laser flow cytometry. Thick sections were enzymatically digested, processed for antigen retrieval and studied for estrogen receptor expression and DNA content (aneuploidy and cell cycle distribution) by flow cytometry. Thin sections cut were stained by immunohistochemistry and studied for ER expression and histopathological grading.

Data from immunohistochemistry and flow cytometry was analyzed for percentage of ER positive cells and DNA content of the subpopulations. Flow cytometric data was compared with immunohistochemical determinations on ER positive or negative status of the tumors examined under a microscope. Correlation between ER expression and the histopathological evaluation of the tumors was undertaken. A manuscript based on these findings is being revised for publication.

2. **Nuclear Volume versus DNA content Analysis of nuclei isolated from Human breast tumors.**

We have recently developed a high-resolution flow cytometer, which can simultaneously measure nuclear volume and DNA content of nuclei in suspension (Appendix I, II, attached). This instrument by using multiparametric analysis of electronic nuclear volume versus DNA content can differentiate between normal and tumor cells and identify tumor cells in secondary sites such as lymph nodes or bone marrow of breast tumor patients. Our first effort was to develop staining methods for optimizing the multiparametric analysis of tumor cells in this instrument. We explored different combinations of DNA binding dye concentrations, pH and tonicity to identify conditions, which provide the best possible data for multiparametric and simultaneous evaluation of nuclear volume and DNA content of nuclei prepared from a tumor biopsy. A manuscript based on these observations was published in Cytometry (Appendix I). To date our data shows that by measuring nuclear volume versus DNA content, we can discriminate between normal and tumor cells present in a breast tumor biopsy. A manuscript based on evaluation of nuclear volume and DNA content of 50 human breast tumor biopsies is being prepared for publication.

KEY RESEARCH ACCOMPLISHMENTS:

- Collection and archiving of 63 breast tumor biopsies and 388-formalin fixed-paraffin embedded blocks of human breast tumors.
- Processing of 75 human breast tumors by flow cytometry and immunohistochemistry for the expression of estrogen receptor expression and DNA content.
- Development of staining methods for simultaneous monitoring of nuclear volume and DNA content of isolated nuclei from solid tumors by high-resolution flow cytometry. Paper published in Cytometry (Appendix I).
- Analysis of 50 breast tumors for nuclear volume and DNA content analysis to detect aneuploidy and discriminate between normal and tumor cells on the basis of electronic nuclear volume versus DNA content measurement (NPE ratio).
- Publication of a paper in Cytometry journal describing the ideal combinations of a fluorochrome, pH and tonicity for multiparametric analysis of human tumors.

REPORTABLE OUTCOMES:

Establishment of the repository for breast tumor biopsies and paraffin embedded blocks needed for the project.

Analysis of 75 breast tumor samples by immunohistochemistry and laser flow cytometry for ER expression and correlation of expression with histopathology.

Development of staining protocols for simultaneous analysis of nuclear volume and DNA content of breast tumor biopsies for the identification of tumor cells and discrimination of normal versus tumor cells based on the nuclear packing efficiency (NPE) ratio.

Analysis of 50 breast tumor biopsies for multiparametric analysis of nuclear volume versus DNA content.

PRESENTATION AT NATIONAL MEETINGS: Data on flow cytometric analysis of breast and other tumors by NASA/ACS flow cytometer for simultaneous monitoring of nuclear volume and DNA content was presented at the Annual Meeting of the American Association for Cancer Research in San Francisco (March 2001).

An abstract describing the use of the multiparametric analysis of nuclear volume and DNA content for analysis of human breast tumors has been submitted for presentation at the annual meeting of the SPIE society in San Jose CA.

CONCLUSIONS: Our data shows that flow cytometric analysis of ER expression in archival formalin fixed paraffin embedded breast tumor samples can be a useful technique for retrospective studies seeking to correlate hormone receptor expression with clinical outcome. The major advantages of the methods we have developed are that they are quantitative and can simultaneously determine ER expression and that of other markers in a heterogeneous tumor cell population.

The staining methods we have developed for simultaneous monitoring of nuclear volume and DNA content of tumor nuclei are capable of discriminating between normal and tumor cells in a heterogeneous tumor sample and possibly can be used for identification of tumor cells in a secondary site such as lymph node or bone marrow.

The combination of nuclear volume measurement with other cellular markers such as DNA and/or hormone receptors by flow cytometry is a powerful technique for analysis of tumor cells.

In the second year of this project we are expanding these techniques for the study of other cellular markers, which are characteristic of tumor cells and can provide information of diagnostic and prognostic significance in study of breast tumors.

APPENDICES:

Appendix I. Wen J, Krishan A, Thomas RA. NASA/American Cancer Society high-resolution flow cytometry project - II. Effect of pH and DAPI concentration on dual parametric analysis of DNA/DAPI fluorescence and electronic nuclear volume. Cytometry 43:12-15. 2001.

Appendix II: Krishan A, Wen J, Thomas RA, Sridhar KS, Smith WI Jr NASA/American Cancer Society high-resolution flow cytometry project - III. Multiparametric analysis of DNA content and electronic nuclear volume in human solid tumors. Cytometry 43: 16-22. 2001.

Cytometry 43:16-22 (2001)

NASA/American Cancer Society High-Resolution Flow Cytometry Project – III. Multiparametric Analysis of DNA Content and Electronic Nuclear Volume in Human Solid Tumors

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Received 8 June 2000; Revision Received 17 August 2000; Accepted 17 August 2000

Background: The NASA/American Cancer Society (ACS) flow cytometer can simultaneously measure electronic nuclear volume (ENV) and DNA content of nuclei. The preceding articles in this volume ("NASA/American Cancer Society High-Resolution Flow Cytometer Project-I") described the schematics, performance, and procedures used for the preparation of nuclei for analysis on this unit. In the present article, we describe the analysis of selected human tumors using the ratio of ENV/DNA content (nuclear packing efficiency [NPE]).

Methods: Tumor specimens (frozen) were minced with scalpels and stained with 1-10 μg/ml of 4',6-diamidino-2-phenylindole (DAPI) dihydrochloride at pH 6.0-7.2. Trout erythrocytes were used as internal standards. Data on ENV and DNA content were collected in list mode files. Propidium iodide-stained nuclei, analyzed on a Coulter XL cytometer, were used for comparison.

Results: Simultaneous measurement of ENV and DNA makes it possible to discriminate between hypodiploid or hyperdiploid tumor cells, as well as to differentiate between near-diploid aneuploid and diploid cells on the basis of their increased ENV. The NPE ratio is a valuable parameter for the detection of small quantities of tumor cells, separating overlapping diploid and aneuploid populations for cell cycle analysis and characterizing the level of differentiation in some tumors.

Conclusion: NPE analysis provides unique measuring capabilities for the study of human solid tumors by flow cytometry. Cytometry 43:16-22, 2001.

Key terms: aneuploidy; DNA content; DNA index; electronic nuclear volume; flow cytometry; hypodiploid; tumors; nuclear packing efficiency; metastasis

Flow cytometry is an important method for the rapid analysis of DNA content and phenotypic markers in cells. Several DNA binding/intercalating fluorochromes (e.g., propidium iodide [PI], 4',6-diamidino-2-phenylindole [DAPI]) are used to directly stain nuclei and to detect aneuploidy and determine cell cycle distribution (1,2). However, due to technical limitations, it is often difficult to detect cells with low levels of aneuploidy (DNA index <1.1) or to detect subpopulations that cannot be distinguished on the basis of their DNA content and/or forward light scatter alone.

In most flow cytometric studies, light scatter (forward angle or 90°) is used for gating (elimination of debris) and selection of subpopulations for DNA and phenotype analysis (3). Although light scatter is related to the size of a cell, it is not a good measure of volume (4-6). In contrast, Coulter principle based on the measurement of impedance is an ideal tool for determination of volume in a flow system (7-9). However, most of the commercially available flow cytometers cannot measure Coulter volume. In the accompanying article (10), we described the NASA/ACS flow cytometer and the use of electronic nuclear volume (ENV) versus DNA for the study of human tissues, calf thymocytes, and trout erythrocytes. Distortions of the nuclear shape, size, and staining variations have been recognized as important diagnostic markers for the identification and classification of human tumors (11). The subjective study of these morphological variables is used

Grant sponsor: American Cancer Society (Florida Division); Grant number: M91-00440.

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in several histological grading systems used in tumor pathology. Simultaneous high-resolution measurement of nuclear volume and DNA content can provide new quantitative information that correlates with morphological parameters used by pathologists for tumor grading.

In this article, we show data on simultaneous measurement of ENV versus DAPI/DNA fluorescence of nuclei from selected human solid tumors analyzed on the NASA/ACS flow cytometer. These data are compared with DNA versus forward angle light scatter of nuclei stained with PI/hypotonic citrate (1) and analyzed on an XL cytometer (Coulter, Hialeah, FL). Data presented show that the ratio of ENV to DNA content (nuclear packing efficiency [NPE]) produces a very characteristic signature that can identify tumor nuclei in a distant metastatic site. This NPE signature may be used to discriminate between normal and tumor cells in a heterogeneous tumor as well as to determine the level of differentiation in a tumor.

MATERIALS AND METHODS

Human solid tumor specimens (approximately 200 samples analyzed to date) obtained from the Departments of Pathology at both the University of Miami and the Suburban Hospital (Bethesda, MD) were stored at -80°C. Sodium citrate and citric acid were purchased from Fisher Scientific (Fair Lawn, NJ). Nonidet P-40 (NP-40) was obtained from Gibco BRL (Grand Island, NY) and DAPI was obtained from Sigma (St. Louis, MO).

DAPI (1 μg/ml) in sodium citrate (1% w/v) containing NP-40 (0.1% v/v), pH 6.0, and NIM-II DAPI, an isotonic medium containing DAPI (10 μg/ml) and 0.6% NP-40, pH 7.2, were used for staining of nuclei analyzed in the NASA/ACS flow cytometer (10). PI (50 μg/ml), pH 6.0, with sodium citrate (1% w/v) and NP-40 (0.1% v/v) was used for the analysis of nuclei in the Coulter XL flow cytometer (1). Nonhematological carcinoma (tumor) specimens were minced with crossed scalpels and stained with DAPI or PI solutions. After pipetting, the resulting suspension was filtered through a 40-μm nylon mesh or 35-μm filters (RATCOM, Inc., Miami, FL). The operational conditions of the instrument and its capability to quantitate DNA at the subpicogram level using trout red blood cells (TRBC) as the internal standard are detailed in the accompanying article (10).

WinMDI (version 2.8, Joseph Trotter, http://facs.scripps.edu) and ModFit (version 5.11, Verity Software House, Topsham, ME) software was used for data analysis and graphics.

RESULTS

Identification of Near-Diploid Tumor Cells

In some tumors, most cytometers can readily identify aneuploid tumor populations by the presence of nuclei with a DNA index (DI) greater than 1.1 but have broad diploid peaks that are suggestive of a possible near-diploid population. Figure 1A,B shows an ovarian tumor that exemplifies this type of histogram. The TRBC internal standard peak in this histogram has a narrow coefficient of

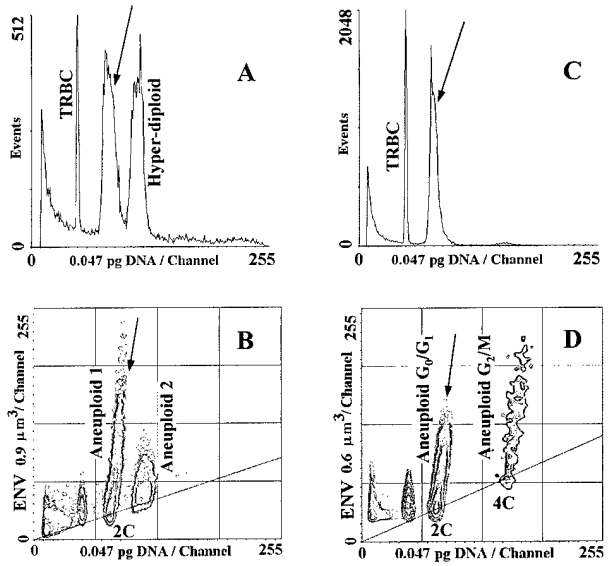


FIG. 1. DNA distribution histograms (**A,C**) and isocontour plots (**B,D**) of an ovarian and prostate tumor sample analyzed on the NASA/ACS flow cytometer.

variation (CV), whereas the G_0/G_1 peak is broad. This suggests the presence of an aneuploid subpopulation (indicated by the arrows), which could not be resolved as a distinct peak. As shown in Figure 1B (ENV versus DNA content), the isocontour plot shows that the nuclei in the near-diploid population have a much larger range of ENV (arrow) than that of the normal diploid G_0/G_1 nuclei. The NPE line for normal human diploid nuclei is shown as the black line passing through the origin. Examples of NPE analysis of normal human diploid nuclei are shown in Figures 3A, 4A, and 7B of the accompanying article (10). There are two distinct aneuploid populations (aneuploid 1 and aneuploid 2), which are identifiable by their NPE signature in this tumor: one with a DI (the ratio of the aneuploid DNA peak channel number to the diploid DNA peak channel number) of 1.05 and the other with a DI of 1.45.

In some tumor populations, the DNA distribution histogram may show a small shoulder on the upper side of the main G_0/G_1 peak, indicating the possible presence of a near-diploid/aneuploid population. The DNA histogram of a prostate tumor (Fig. 1C) has this characteristic. In Figure 1D (the isocontour plot of ENV versus DNA), the nuclei associated with the shoulder on the DNA histogram (arrow) have elevated ENV displaying an NPE signature characteristic of tumor cells. The line connecting the G_0/G_1 (2C) and the G_2/M (4C) populations is the NPE line for normal human cells (10).

Using DNA histograms alone (Fig. 1A,C), it is difficult to identify the near-diploid/aneuploid populations. However, in the two-parameter isocontour plots (Fig. 1B, D), these populations are identifiable as having nuclei with a three to fourfold greater ENV and a DI of 1.05. The

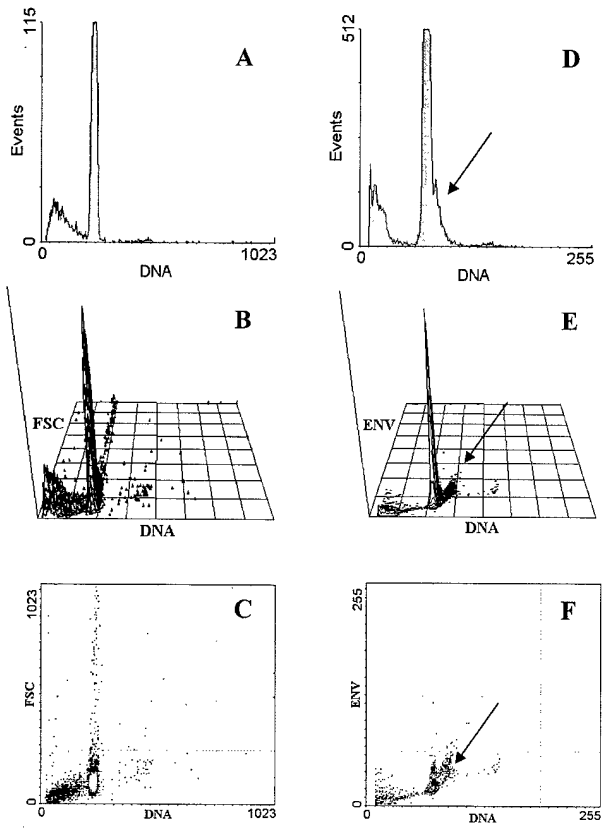


FIG. 2. **D–F:** A small population of nuclei with aneuploid DNA content (arrows) was seen in cells from the pleural fluid of a mesothelioma patient using the NASA/ACS cytometer. **A–C:** Cells stained with PI and analyzed on a Coulter XL cytometer did not detect the presence of the aneuploid population. ENV versus DNA plots (E,F) show elevated ENV for this aneuploid population.

distinctive NPE signature confirms the presence of aneuploidy in these tumors.

Detection of Small Aneuploid Populations

The ENV versus DNA plots combined with high-resolution histograms can often identify small aneuploid tumor populations not detectable or interpretable by the analysis of DNA versus forward scatter alone. Figure 2 shows DNA histograms (Fig. 2A,D), three-dimensional isometric plots (Fig. 2B,E), and dot plots (Fig. 2C,F) of nuclei from the pleural fluid of a mesothelioma patient analyzed on the Coulter XL flow cytometer (Fig. 2A–C) and the NASA/ACS cytometer (Fig. 2D–F). Most of the cells in this effusion are included in a single diploid peak when examined in the XL cytometer (Fig. 2A–C). In contrast, DNA histograms generated on the NASA/ACS cytometer (Fig. 2D) identified a small but distinct peak of aneuploid tumor cells with a DI of 1.05 (arrow). The use of ENV versus DNA content (Fig. 2E,F) shows much more heterogeneity; the arrows point to the presence of the subpopulation with aneuploid DNA content and a distinct ENV profile. It is important to note that the small aneuploid population identified in Figure 2D has nuclei with an ENV twofold greater than that of the diploid nuclei (Fig. 2E,F).

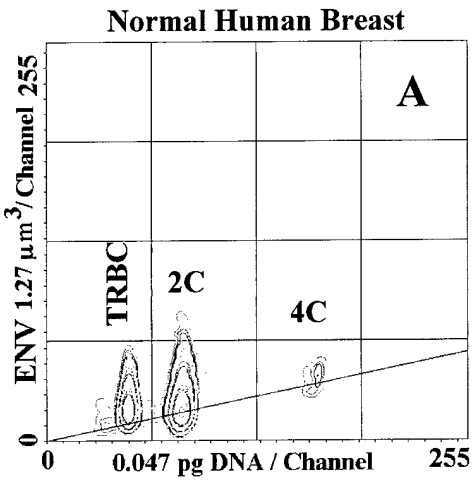
Identification of Metastatic Tumor Cells

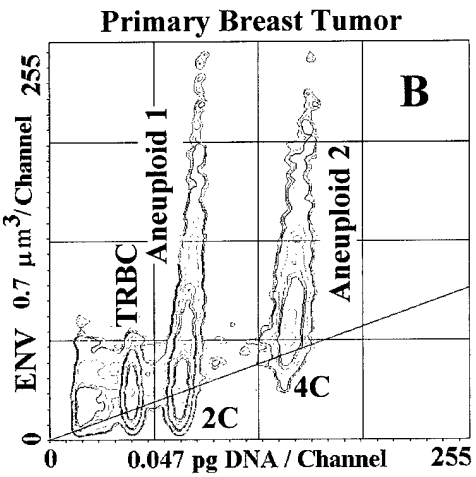
The use of ENV versus DNA can often be used to determine the presence of aneuploid tumor cells in metastatic tumors. Figure 3 shows the ENV versus DNA content of normal breast tissue (Fig. 3A), a primary breast tumor (Fig. 3B), and a lymph node with histopathologically confirmed tumor infiltration (Fig. 3C). A reference line drawn across the plots represents the NPE value of normal nuclei and connects the center of the diploid G_0/G_1 (2C) and G_2/M peaks (4C; 10).

Nuclei from the normal breast tissue (Fig. 3A) are characterized by a small ENV signal (confined within the 1st quadrant of the Y-axis) and a pattern similar to that of cells from normal human breast, colon, small intestine, thyroid, lymph node tissue, and lymphocyte samples (10). Nuclei from these normal tissues had similar low ENV with a mean volume of $21 \pm 0.5\ \mu m^3$. In contrast, the ENV signal of the aneuploid nuclei (DI of 1.06) from the primary breast tumor (Fig. 3B, Aneuploid 1) was spread over four quadrants of the Y-axis. Figure 3B also shows the presence of a near-tetraploid population (Aneuploid 2) with high ENV and a DI of 1.75. The ENV pattern seen in this tumor was characteristic of several other poorly differentiated breast tumors studied (data not shown).

Figure 3C shows nuclei isolated from a lymph node of the node-positive patient whose primary tumor is shown in Figure 3B. The lymph node of this patient was histopathologically confirmed to contain tumor cells. A predominant population of diploid nuclei and a smaller population of nuclei with G_2/M DNA content lie along the normal tissue NPE line. Nuclei from normal human lymph nodes (as shown in Fig. 8B of the accompanying article,10) have in general a small ENV signal confined within the 1st quadrant of the Y-axis. The diploid G_0/G_1 and G_2/M nuclei in this lymph node are on the NPE line for normal human diploid nuclei and stand distinct from the Aneuploid 1 and Aneuploid 2 nuclei with larger ENV. A comparison of the aneuploid populations of the primary tumor (Fig. 3B) with the aneuploid populations in the lymph node (Fig. 3C) shows that the distinct NPE pattern seen in the primary tumor was also seen in the lymph node. The population marked Aneuploid 2 in Figure 3C (representing 3% of the total population) illustrates the sensitivity of the ENV versus DNA method for distinguishing between normal diploid G_2/M and near-tetraploid tumor cells and for detecting small number of aneuploid nuclei in a tumor.

ENV versus DNA analysis can also be used for detecting the presence of tumor cells in the surgical margins and distant metastasis of a tumor. Figure 4 shows a comparison of ENV/DNA content in nuclei from normal colon (Fig. 4A), in primary adenocarcinoma of the colon (Fig. 4B), and in distant metastasis of this tumor (Fig. 4C). The population of diploid nuclei is characterized by a small ENV signal (confined within the 1st quadrant of the Y-axis) with a prominent G_2/M (4C) population falling on





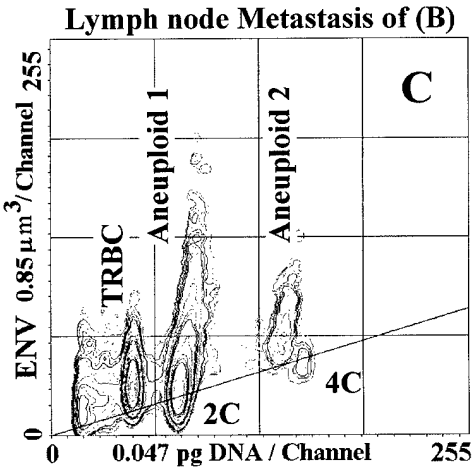
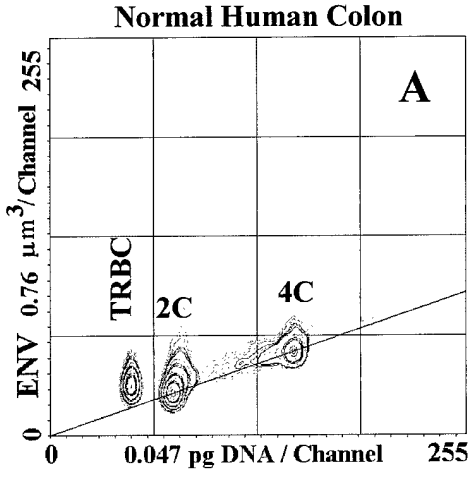
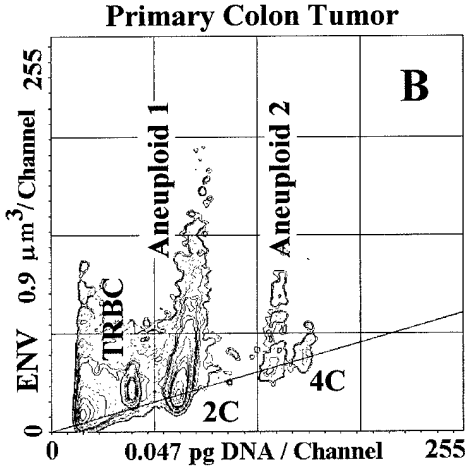


FIG. 3. Using NPE analysis, a comparison of normal human breast tissue (**A**), a primary poorly differentiated breast carcinoma (**B**), and the metastasis of the primary to a distant lymph node (**C**) is shown. The aneuploid populations have elevated ENV values and are distinct from the diploid populations (2C and 4C). The unique NPE pattern seen in the primary tumor is evident in cells from the metastasis (C).





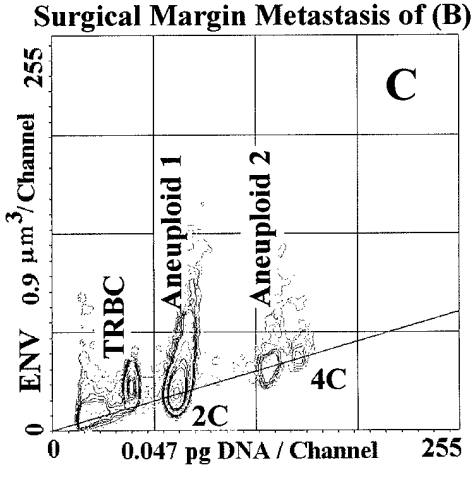


FIG. 4. Using NPE analysis, a comparison of normal human colon tissue (**A**), a primary poorly differentiated adenocarcinoma of the colon (**B**), and the metastasis of the primary to the end of the surgical resection (**C**) is shown. The aneuploid populations contain elevated ENV values and are distinct from the diploid populations (2C and 4C). The unique NPE pattern of the primary tumor was also evident in the metastasis. The population labeled "Aneuploid 2" in Figure 4C represents 3% of the total sample.

the normal tissue NPE line. Figure 4B of the primary tumor shows a near-diploid/aneuploid population (Aneuploid 1; DI of 1.09) with very large nuclei and a second smaller near-tetraploid population (Aneuploid 2) with a DI of 1.75, which is clearly distinguished by its ENV. Figure 4C shows the tissue from the end portion of the surgical resection of the patient's primary tumor shown in Figure 4B. A comparison of the aneuploid populations of the primary tumor (Fig. 4B) with the aneuploid populations in the colon resection (Fig. 4C) shows that the distinct NPE pattern seen in the primary tumor was also seen in the secondary site. The approximately 3% tumor cells in this resection were not detected in the initial histological examination. However, subsequent reexamination confirmed the presence of tumor cells at this site.

Differentiating Hypodiploid From Hyperdiploid

ENV versus DNA analysis can often facilitate discrimination between hypodiploid and diploid cells in a population. Figure 5A shows the DNA histogram of a melanoma with a hypodiploid shoulder (arrow). Figure 5B shows that the nuclei with the larger ENV are from the population containing hypodiploid DNA content with a DI of 0.94. The large population of hypotetraploid nuclei with elevated ENV (Fig. 5B) further confirms this. The normal tissue NPE line passes through the center of the diploid (2C) and the small diploid G_2/M (4C) population.

Poorly Differentiated Versus Well-Differentiated Tumors

Figure 6 shows data from two well-differentiated breast carcinomas. The tumor in Figure 6A has a predominant hyperdiploid population (DI of 1.4). The tumor in Figure 6C has a small hyperdiploid population (DI of 1.25). A comparison of these well-differentiated breast tumors with the poorly differentiated breast tumor (Fig. 4B,C) and the poorly differentiated ovarian, prostate, and colon tumors (Figs. 1, 3) shows that the ENV in these well-differentiated tumors was smaller than that of the poorly differentiated tumors. The normal tissue NPE line is labeled "1" (Fig. 6B,D). There is a small shift in the NPE values for both of these tumors as shown by the line labeled "2" (Fig. 6B,D). In Figure 6D, the diploid G_2/M (4C) population lies along line 1 and the aneuploid G_2/M population lies along line 2. In Figure 6B, the aneuploid G_2/M population lies along line 2, but there is no discernible diploid G_2/M population present.

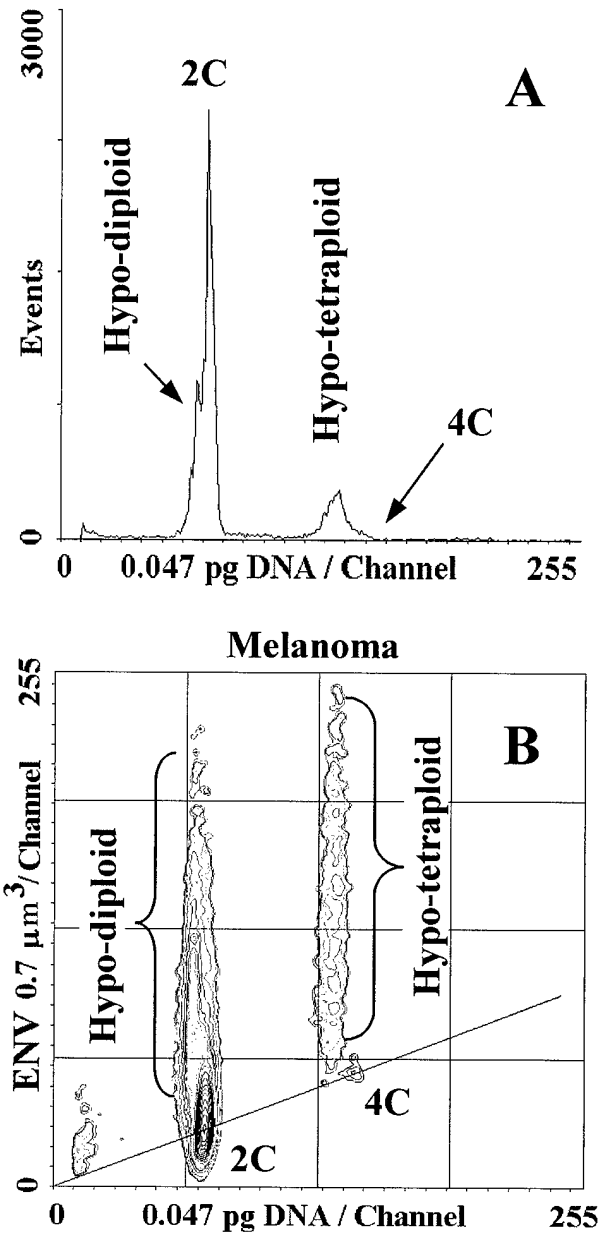


FIG. 5. The ability to differentiate between hypodiploid and hyperdiploid tumors is shown. **A:** DNA histogram of a hypodiploid tumor. The arrow indicates the hypodiploid population. **B:** It may be identified as such by the ENV versus DNA (NPE signature) analysis. The large nuclei have lower DNA content and are distinct from the diploid populations (2C and 4C).

DISCUSSION

One of the major uses of flow cytometry has been for the quantitation of DNA (cell cycle analysis) and the detection of aneuploid tumor cells (12). Fluorochromes such as PI (1), acridine orange, mithramycin, and DAPI (2) have been used for this purpose. The NASA/ACS high-resolution flow cytometer can reproducibly identify cells with a DI as low as 1.02 (10). By combining ENV with the DNA measurement, this study has shown that subpopulations, which are otherwise difficult to detect in heterogeneous populations of nonhematological carcinomas, can be identified.

The basic concept of measuring the volume of cells suspended in a conducting fluid (electronic cell volume [ECV]) is described in a U.S. Patent issued to Wallace H. Coulter in 1953 (7). An electric field is applied across the aperture, which causes current to pass through the flow chamber. As the particle passes through the aperture, a measurable decrease in current, proportional to the vol-

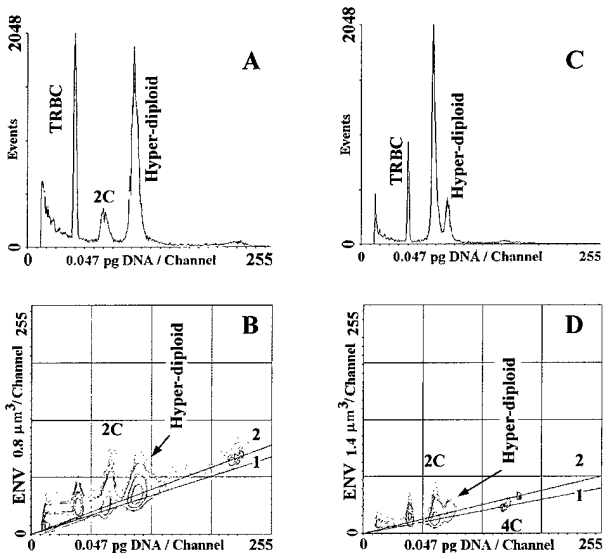


FIG. 6. DNA histograms (**A,C**) and the NPE analysis (**B,D**) of two well-differentiated breast carcinomas. Comparison with the poorly differentiated tumors in Figures 1, 3, and 4 demonstrates the ability of NPE analysis to characterize the state of differentiation.

ume of the particle, occurs. For a variety of technical reasons, it has proven difficult to design a flow chamber that combines simultaneous Coulter volume and optical measuring capabilities. Therefore, the commercially available flow cytometers in general do not have the capability to measure electronic volume. They use forward scatter cone (FSC) of light scattering and time of flight to approximate the diameter of a cell as it passes through a beam of light. FSC can determine the diameter of spherical particles that have a large index of refraction with respect to their surrounding medium and no internal structure. Some research cytometers and one commercially available flow cytometer have Coulter volume measuring capabilities that use large-diameter apertures to decrease clogging and employ down stream optical measurements. These units are suitable for whole cells, but do not readily detect human nuclei with diameters in the 3-4 μm range. Decreasing the aperture size to 50 μm would increase sensitivity but is not practical, as it would result in an unacceptable rate of clogging. The large amount of cell debris that accompanies nuclear isolation preparations presents difficulties to the ECV analog-to-digital converter (ADC). Without the ability to gate using the DNA measurements, the ADC will spend the majority of its conversion time working on cell debris rather than nuclei. The triangular aperture geometry has the sensitivity of a 50-μm aperture without the associated clogging problems (10). The simultaneous nature of the DNA and ENV measurements makes gating of the cell debris practical using the DNA fluorescence signal.

The lack of a strong linear relation between FSC and particle size outside the range of 10-20 μm makes it relatively difficult to use this parameter for the identification of nuclear subpopulations that are in the 3-4-μm range (4,5,13). As shown in this study, forward angle light scatter does not identify nuclear subpopulations that can be readily identified on the basis of their ENV, primarily because of the large CV of the FSC measurement when analyzing biological material (10).

The theoretical relationship between the change in current observed when a particle traverses the measuring chamber and the volume of that particle is discussed by Grover et al. (14). The main assumption in the derived relationship is that resistivity of the particle must be greater than that of the suspending media. An examination of the equations derived by Grover et al. (14) shows that a 10% error in the observed volume will occur when the ratio of the particle to media resistivity is 4:1. The error diminishes to 1% at a ratio of 50:1. An examination of Figures 3C,3D of the accompanying article (10) provides information as to this ratio for our nuclear preparations. The light scatter method for measuring the diameter of trout erythrocyte nuclei (TRBC) gave a mean diameter of 3.55 ± 0.14 μm. The ENV measurement on the same sample gave a value of 3.67 ± 0.03 μm. The ENV diameter was derived from the volume measurement using a spherical model of the TRBC nucleus. The ENV value is within the experimental error of the FSC value. These data suggest that the error in the Coulter volume of the nuclei has a value somewhere between 0.2-7%.

This study shows that two-parameter analysis of ENV versus DNA content can be a valuable tool for the analysis of human tumors. The data presented show that nuclei with near-diploid DNA content contain very large nuclei and are easily identified as being distinct from diploid nuclei by their ENV versus DNA ratio. This method can be used for identifying near-diploid or near-tetraploid aneuploid nuclei and for differentiating hypodiploid from hyperdiploid tumors. The separation of the diploid from the aneuploid populations that occurs will allow better analysis of each compartment of the diploid and aneuploid portions of the tumor, providing a better estimate of the S phase of the tumor.

The NPE analysis shows an ability to detect tumor cells in low concentration (i.e., 3%) in primary sites, tumor margins, and in secondary locations, such as lymph nodes and distant metastatic sites. These populations, which could not be identified on the basis of their light scatter and DNA content, can be readily identified by their NPE ratio. The NPE ratio may also allow us to distinguish between tumors with different stages of differentiation. Preliminary data (not shown) indicate that NPE analysis may be of value for the analysis of hematological tumors.

Aneuploid populations in the bivariate distribution plots of ENV versus DNA show a definite slope. This incline suggests the existence of a positive correlation between the ENV and the DNA content of these nuclei. Our preliminary data show that benign tumors have nuclei with elevated ENV. However, these populations do not show a similar inclined slope. As the slope of the aneuploid populations seems to be dependent on dye concentration reaching a plateau at 5 μg/ml of DAPI (unpublished data), it is possible that tumor cell nuclei with

increased ENV bind more of the dye due to differences in chromatin structure.

Ongoing work in our laboratory is focused on the monitoring of hormone receptor expression in human prostate and breast tumors (15,16) and their relationship with NPE as assessed with the high-resolution and ENV measuring capabilities of the NASA/ACS flow cytometer.

ACKNOWLEDGMENTS

We gratefully acknowledge the help of Mr. Wei You, Ms. Ilia H. Andritsch for their help in the preparation and analysis of the samples, and Mr. Scott Adler for his hospitality and help in collection of tumor samples.

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Cytometry 43:12-15 (2001)

NASA/American Cancer Society High-Resolution Flow Cytometry Project – II. Effect of pH and DAPI Concentration on Dual Parametric Analysis of DNA/DAPI Fluorescence and Electronic Nuclear Volume

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Received 8 June 2000; Revision Received 13 September 2000; Accepted 5 October 2000

Background: In the present paper, we describe the effect of 4', 6-diamidino-2-phenylindole (DAPI) dihydrochloride concentration and pH on the resolution of DNA distribution histograms generated by dual-parametric simultaneous analysis of DNA content and electronic nuclear volume (ENV).
Methods: Nuclei from tissue culture cell lines and frozen human solid tumors were isolated in nuclear isolation media containing different concentrations of DAPI, at various pH levels, and analyzed on a NASA/American Cancer Society (ACS) flow cytometer. Samples stained with propidium iodide/hypotonic citrate and analyzed in a Coulter XL flow cytometer were used for comparison.
Results: Nuclei stained with DAPI concentration of 1-3 μg/ml, pH 6.0, gave the best resolution for the detection of the near-diploid and near-tetraploid populations. Simultaneous use of ENV and DAPI/DNA fluorescence under these conditions identified subpopulations that otherwise could not be detected by DNA analysis alone.
Conclusions: Staining at 1-3 μg/ml DAPI, pH 6.0, was optimal for the detection of aneuploid populations, especially the near-diploid and/or near-tetraploid populations in human tumors. Cytometry 43:12-15, 2001.

Key terms: DAPI; aneuploidy; flow cytometry; pH; DNA index; electronic nuclear volume; fluorochromes

Flow cytometric analysis of cellular DNA content (for the identification of aneuploid populations and cell cycle distribution) and the expression of phenotypic markers are of diagnostic and prognostic value in the management of human tumors. Different DNA-binding fluorochromes (e.g., propidium iodide [PI] and 4', 6-diamidino-2-phenylindole [DAPI] dihydrochloride) have been used to stain nuclei, detect aneuploidy, and determine cell cycle distribution (1-3).

Detection of cells with low levels of aneuploidy (DNA index [DI] <1.1) on the currently available commercial flow cytometers can prove problematic (4). A flow instrument developed by RATCOM, Inc. (Miami, FL) with the support of NASA/American Cancer Society (ACS; Florida Division) uses a unique triangular flow cell to simultaneously measure electronic nuclear volume (ENV; based on the Coulter principle) and DNA fluorescence (5,6). In an accompanying paper, we described the engineering and performance characteristics of this instrument, which has the ability to resolve populations with a DI of 1.02 on a routine basis (5).

In the present study, we describe a sample preparation and staining method used for simultaneous analysis of ENV and DNA content of tissue culture cells and fresh/frozen solid human tumors in the NASA/ACS flow cytometer. The effect of pH and fluorochrome concentration on the coefficient of variation (CV) of the G_0/G_1 peak (in DNA the distribution histograms) is described. These data are compared with the DNA content of nuclei stained with PI/hypotonic citrate and analyzed on a Coulter XL cytometer. In an accompanying paper, we used these procedures to analyze a number of human tumors for the simultaneous determination of DNA content and ENV (6).

Grant sponsor: American Cancer Society, Florida Division; Grant number: M91-00440; Grant sponsor: Department of the Army; Grant number: DAMD17-00-1-0342.

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MATERIALS AND METHODS

Tumor and Cell Lines

The mouse P388/R84 cell line was cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS). Short-term cultures of a murine cell line (MAT-B1) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS. Human solid tumor specimens (obtained from the repository at the Department of Pathology, University of Miami; Cooperative Human Tissue Network, Columbus, OH; and Suburban Hospital, Bethesda, MD) were stored at -80°C. Sodium citrate and citric acid were purchased from Fisher Scientific (Fair Lawn, NJ). Nonidet P-40 (NP-40) was obtained from Gibco BRL and DAPI dihydrochloride from Sigma (St. Louis, MO).

Staining Solutions

The following DAPI and PI staining solutions were prepared: (1) DAPI solutions at different pH levels: DAPI (1 μg/ml) was mixed in sodium citrate (1% w/v) containing NP-40 (0.1% v/v) at pH levels 4.0, 5.0, 6.0, 7.0, 8.0. (2) DAPI solutions at different concentrations (0.1, 0.5, 1, 3, 5, 7, 10 μg/ml), pH 6.0, were mixed with 1% sodium citrate and 0.1% NP-40. (3) PI solution (1, 5, 10, 25, and 50 μg/ml) at different pH levels (4.0, 5.0, 6.0, 7.0, 8.0) in sodium citrate (1%) containing NP-40 (0.1% v/v). Citric acid (1% w/v) or NaOH (1 M) was used to adjust the pH level.

Nuclear Isolation and Staining

P388/R84 and MAT-B1 suspension cell cultures (about 1×10^6 cells) were harvested by centrifugation at 600 × g for 5 min. The cell pellets were washed once with 1 × phosphate-buffered saline (PBS) and stained with DAPI or PI solution (1 ml) for 10 min at 4°C.

Tumor specimens stored at -80°C were minced with crossed scalpels and aliquoted into different DAPI or PI solutions. The resulting suspensions were filtered through 35-μm filters (RATCOM). The use of trout red blood cells (TRBC) and operational conditions of the instrument were as stated in the accompanying paper (5). DAPI-stained samples were analyzed on the NASA/ACS flow cytometer and PI-stained samples on a Coulter XL cytometer. Both instruments were calibrated and performance optimized by a company-trained technician. Cell cycle analysis of the DNA histograms was performed by Modfit version 5.11 (Verity Software House, Topsham, ME). Multiparameter graphics were prepared using WinMDI (version 2.7, Joseph Trotter, http://facs.scripps.edu).

RESULTS

Effect of pH on CV and Debris

Figure 1 shows typical data analysis from the highly proliferative P388/R84 cell line and the short-term culture of MAT-B1 cells with a low proliferation index. Both cell lines were stained with DAPI solutions, pH 5–8. The lowest CV (2.45–2.5%) for G_0/G_1 peaks and the least amount of debris was seen at pH 6 in both cell lines. An

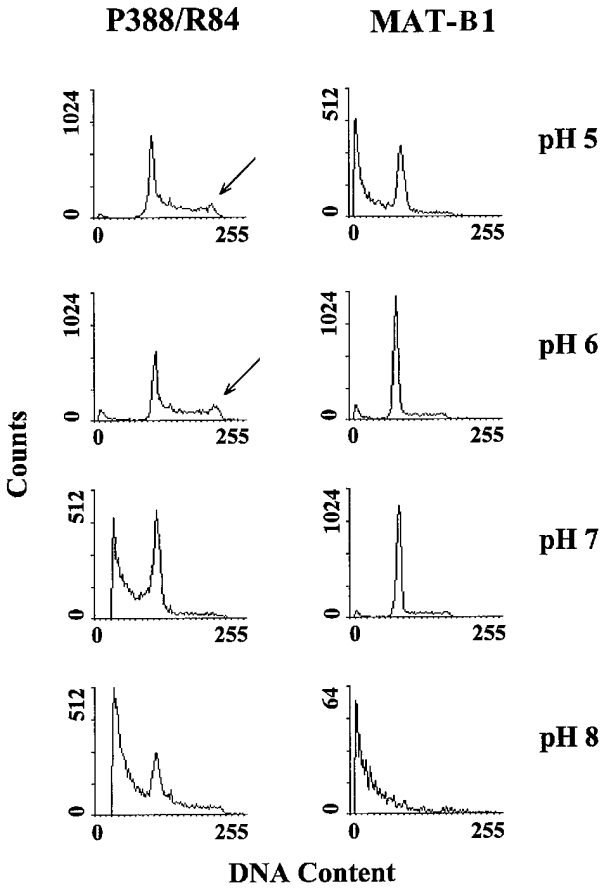

FIG. 1. Effect of pH on DNA histograms. Mouse P388/R84 and MAT-B1 cells were stained with DAPI solutions (1 μg/ml) at pH 5–8 and analyzed on the NASA/ACS flow cytometer.

increase in debris and degradation of resolution were seen in cells stained at pH 8. In P388/R84 cells stained at pH 7 or 8, the loss of the prominent G_2/M peak (seen in DNA histograms of cells stained at pH 5 or 6, arrows) combined with an increase in debris (defined as material with less then G_0/G_1 DNA content) was seen.

Effect of DAPI Concentration on CV and Mean Fluorescence Channel (MCF)

Concentration of DAPI had a major effect on the CV and MFC of the G_0/G_1 peak in the three tumors tested (Fig. 2). In contrast, the CVs in TRBC did not significantly change over the DAPI concentration ranging from 1 to 10 μg/ml (Fig. 2, first column). In P388/R84, MAT-B1, and breast tumor cells, the best CVs (3.1%, 2.3%, and 1.9 %, respectively) were seen at DAPI concentration of 1–3 μg/ml. An increase in DAPI concentration increased CVs, resulting in loss of discrimination of the aneuploid peaks in the breast tumor sample. When breast tumor nuclei stained with DAPI (10 μg/ml) were resuspended in DAPI-free citrate buffer and reanalyzed, the CVs of the G_0/G_1 peaks improved and was as low as that of nuclei stained with 1–3 μg/ml of DAPI (data not shown). In samples stained with

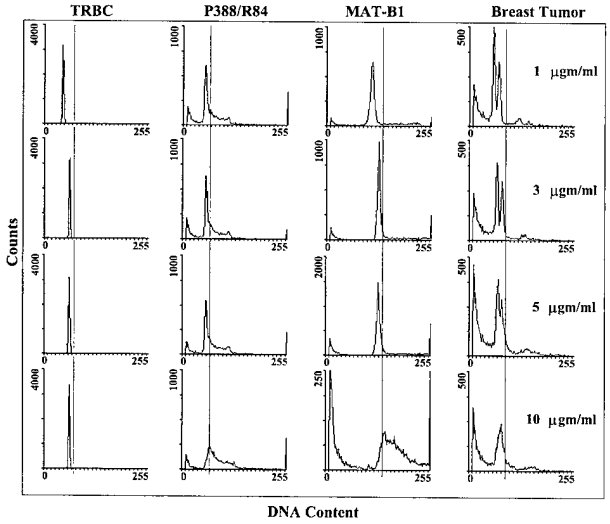

FIG. 2. Effect of DAPI concentration on DNA histograms of TRBC, P388/R84, MAT-B1, and nuclei isolated from a human breast tumor sample.

DAPI (1 μg/ml) and stored at 4°C in dark, no deterioration or change in DNA histograms was observed for up to 72 h of storage (data not shown).

Aneuploidy Detection in Cells Stained With DAPI or PI

Figure 3 compares the DNA histograms of three different human breast tumor specimens stained with the PI/citrate method (analyzed on a Coulter XL flow cytometer) with cells stained with DAPI solution (1 μg/ml, pH 6.0) and analyzed on the NASA/ACS cytometer. In cells stained with the PI/hypotonic citrate method (1; pH 4-8, PI concentrations 10-75 μg/ml), no significant changes in CVs were detected (data not shown). In general, DAPI-stained nuclei (Figs. 3A-C) had three to fourfold narrower CV than PI-stained nuclei (Figs. 3D-F). Figures 3A,D show that the DNA histograms of a breast tumor specimen stained with DAPI or PI were essentially similar, showing

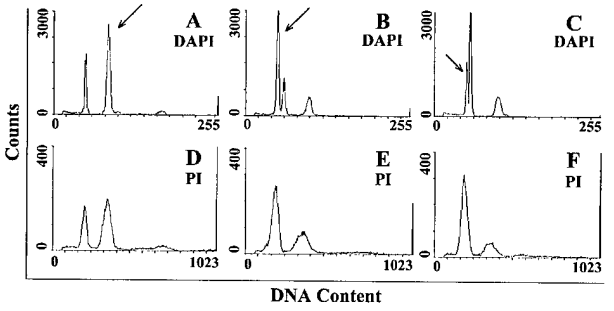

FIG. 3. DNA histograms of nuclei from three human breast tumors analyzed on the NASA/ACS flow cytometer (A–C) and a Coulter XL (D–F). CVs of DAPI-stained samples analyzed on the NASA/ACS flow cytometer were 3.24, 3.17, and 2.96. Those of the PI-stained samples analyzed on the XL were 9.82, 9.94, and 10.32, respectively. Aneuploid subpopulations are indicated by arrows.

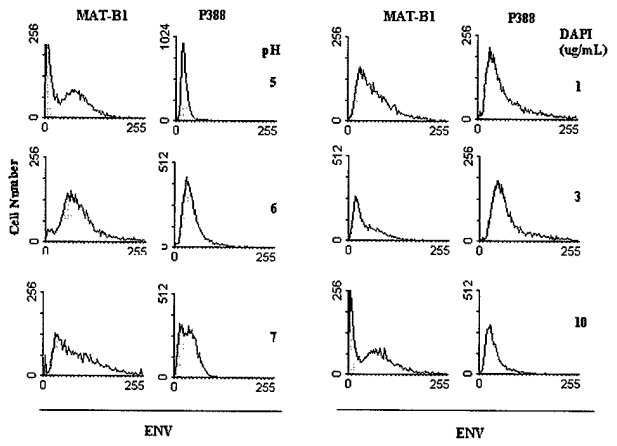

FIG. 4. Effect of pH 5-7 and DAPI concentration on ENV of MAT-1B and P388/R84 cells.

a major aneuploid population (arrow), except for the narrower CV in the DAPI-stained sample. However, the improved CVs of DAPI-stained samples did identify aneuploid (near-diploid) peaks in the other two human breast tumor samples as shown in Figures 3B,C (arrows). In samples stained with PI solution (50 μg/ml PI, 1% Na citrate, 0.1% NP-40, pH 6.0) and analyzed on the Coulter XL, these near-diploid aneuploid populations could not be distinguished from the diploid peaks (Figs. 3E,F). The aneuploid peaks identified in Figures 3B,C had a DI of 0.83 and 0.89, respectively.

Effect of DAPI Concentraton and pH on ENV Measurement

Figure 4 shows the effect of pH (5-7) and DAPI concentration on the ENV of MAT-1B and P388/R84 cells. A broadening of the ENV distribution in both cell types was seen with an increase in pH from 5 to 6. However, in cells stained at pH.6.0, a distinct and well-formed ENV distribution histogram was obtained. Increase in concentration of the DAPI from 1 to 10 μg/ml decreased the peak channel value of ENV in both cell types. These data showing that DAPI concentration of 1-3 μg/ml and pH of 6.0 was ideal for dual-parametric analysis were confirmed in our analysis of human tumors described in the accompanying publication (6).

DISCUSSION

DAPI has been widely used for flow cytometric determination of DNA content and cell cycle analysis (2,3,7,8). Kapuscinski (2) described the use of DAPI for DNA analysis, identification of mycoplasma, and for staining a variety of biological materials. DAPI binds to DNA to form a stable complex in the minor groove of the A-T sequence (8). The amount of A-T base content, the degree of chromatin condensation, and the presence of anionic detergents have been shown to affect DNA/DAPI fluorescence (2). Taylor and Milthorpe (9) reported that DAPI/DNA fluorescence varied in different cell types. In mouse cells,

low-affinity binding sites were not stained at low DAPI concentrations (0.3 μg/ml). Hedley et al. (10) reported that staining with 1 μg/ml of DAPI in RPMI 1640 medium for 30 min gave the best CV. Darzynkiewicz et al. (11) showed that removal of histones by washing with 0.1 N hydrochloric acid increases DAPI binding to DNA by 45%. They reported that a 20-fold increase in quantum yield of DAPI bound to DNA compared with the unbound dye. In cells, dye equilibrium was reached in 5 min. They also showed that differentiated cells had 8% lower DAPI fluorescence than cells from log-phase cultures and that the DAPI fluorescence was not affected by the removal of histones (11). Teodori et al. (7) used 2 μg/ml of DAPI in combination with a protein dye (sulforhodamine) to detect aneuploid tumor cells that could not be detected in single-parameter histograms of DAPI fluorescence.

Data in the present report show that for analysis in the NASA/ACS flow cytometer, a DAPI concentration of 1–3 μg/ml, pH 6.0, gave the best CV for the G_0/G_1 peak. DAPI concentrations higher than 3 μg/ml decreased resolution, possibly due to oversaturation (or binding to other low-affinity sites). This loss of resolution was reversible by dilution of the stained nuclei with DAPI-free medium.

There are not many reports on the effect of pH on DAPI-DNA binding. Smith et al. (12) reported that the fluorescence (violet and green) intensities of Hoechst 33342-DNA were significantly changed over a pH range of 3–11.5. Our data show that the pH of the DAPI staining solution is an important parameter. Staining at pH 6.0 gave the best overall results as determined by the reduced amount of nuclear debris, the smaller CV of the G_0/G_1 peak, and preservation of the G_2/M peak.

We conclude that for the optimal staining of DNA with DAPI, the staining solution should contain 1–3 μg/ml of dye concentration at pH 6.0. The use of DAPI for DNA content analysis provided resolution of aneuploid populations that was not possible with the PI staining protocol routinely used in this laboratory (1).

When used for simultaneous analysis of DAPI-DNA fluorescence and ENV, this staining protocol allows for the detection of subpopulations that cannot be identified by analysis of DNA content alone or in combination with light scatter. The presence of near-diploid and/or near-tetraploid aneuploid populations could be either related to differences in the amount of DNA or may be related to biological variations alluded to in earlier work on PI and DAPI-stained cells (11,13). The accessibility of the binding sites due to chromatin structure changes and/or gene mutations (multiple point mutations, deletion, and insertion) may account for the observed variations.

Our studies show that samples stained by this procedure could be prepared in less than 5 min and were stable for at least 72 h when stored at 4°C in the dark. This is especially important for clinical studies when it may not be possible to carry out same-day analysis.

ACKNOWLEDGMENTS

We thank Dr. William Smith (Suburban Hospital, Bethesda, MD) for providing the human tissue specimens and their histopathological evaluation.

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