

In Situ Hybridization and Image Analysis on Nuclei Sorted on the Basis of DNA Content

Introduction

Many investigators have used flow cytometry to classify abnormal cell populations in tumors by measuring the DNA content of each cell.^{1,2} Flow cytometry allows an investigator to evaluate large numbers of cells or nuclei, typically from 10,000 to 50,000, in a relatively short time. When a population with abnormal DNA content is found, it might be desirable to sort the cells for further analysis. The use of a cell sorter makes it possible to isolate populations of interest to further characterize the cells based on morphology or other properties. This application note details the use of the FACSort™ for sorting nuclei based on DNA ploidy, and demonstrates the suitability of the sorted nuclei for morphologic analysis by microscopic examination or for in situ hybridization with chromosome-specific probes.*

* For Research Use Only. Not for use in diagnostic or therapeutic procedures.

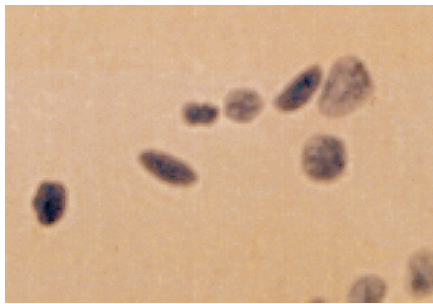


Figure 1a

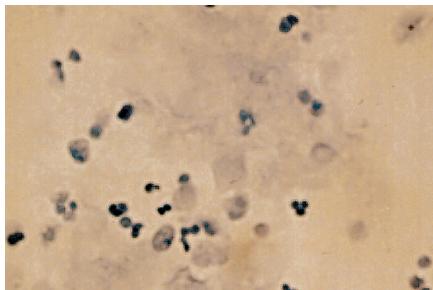


Figure 1b

Figure 1 Feulgen Azure A-stained preparation of nuclei sorted on the basis of DNA content. (1a) Normal staining. (1b) Preparation showing excessive autolysis.

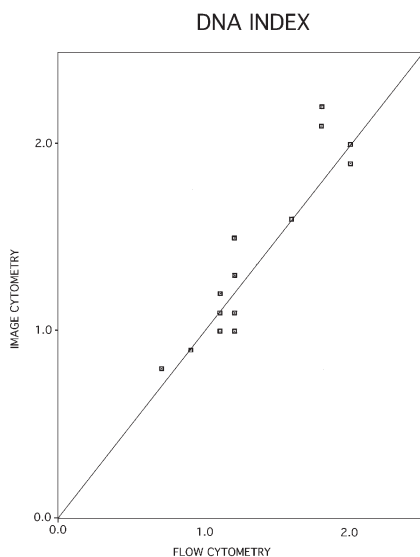


Figure 2 DNA index as determined by flow cytometry versus DNA index as determined by image cytometry for several samples. The diagonal line is a theoretically correct line where both measurements are the same.

Materials and Methods

DNA Staining and Flow Cytometry

Nuclei from approximately 10^6 cells from each solid tumor or bladder washing were prepared and stained³ with propidium iodide (PI) using the CycleTEST™ DNA Reagent Kit (BD Biosciences, Erembodegem-Aalst, Belgium). After centrifugation, 250 μ L of solution A was added to the cell pellets and incubated for 10 minutes at room temperature. After incubation, 200 μ L of solution B was added to each tube and the tube was gently vortexed. After a second 10-minute incubation at room temperature, 200 μ L of ice-cold solution C was added. Samples were gently mixed and filtered through a 50- μ m Nitex™ nylon mesh (Amlyabo, Chassieux, France).

Propidium iodide fluorescence was collected in the FL2 channel of the FACSsort, with the amplifier set to linear. The pulse width and area were used to identify singlets. Data was acquired on a FACSsort with LYSYS™ II software and analyzed using CellFIT™ software.

Sorting

For sorting, the gate was set in an FL2-Area histogram on the G_0/G_1 population of a defined DNA aneuploid peak. Diploid was defined by a reference to a chicken red blood cell control. Whenever available, normal tissue adjacent to the tumor was also used to identify the location of the diploid G_0/G_1 peak. The sort mode was set to Exclusion, and the flow rate was set to either LO or MED. Approximately 45 mL of cells were collected into 50-mL centrifuge tubes coated with bovine serum albumin.* Refer to the *FACSsort User's Guide*, Chapter 3.

Immediately after the sort, 5 mL of a buffered 10% formaldehyde solution (Carlo Erba, Paris, France) was added to each tube, and the cells were gently mixed and centrifuged at 4,000 $\times g$ for 10 minutes. The pellet was resuspended in 100 μ L of phosphate-buffered saline (PBS) and cytospin-centrifuged at 1,300 rpm (2,000 $\times g$) for 5 minutes to prepare slides. At least 50,000 nuclei were necessary to obtain a suitable slide.

Morphological Examination and Image Analysis

Slides were stained using a Papanicolaou procedure⁴ to assess morphology. For image analysis, slides were stained with Feulgen Azure A using the CAS 100 Staining Kit (Cell Analysis Systems, Inc, Elmhurst, IL). DNA quantification was performed on a CAS 200 system.

In Situ Hybridization

Slides were incubated in a pepsin solution (40 mL 0.001 N HCl, 2 mg pepsin) for 25 minutes at 37°C. Chromosome-specific α -satellite DNA probes (Oncor, Gaithersburg, MD) for chromosomes 1, 7, and 17 were used in all cases, and probes for 9 and 11 were used only in selected cases, following the manufacturer's instructions. The probes were detected using a fluorescence-based detection system, the Oncor Chromosome in situ Kit.

* US Patent No. 5,232,828

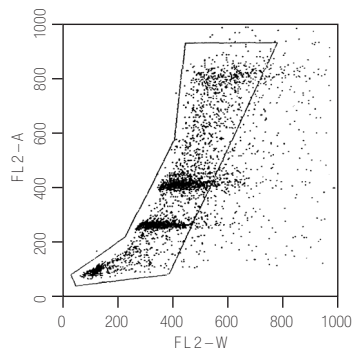


Figure 3a

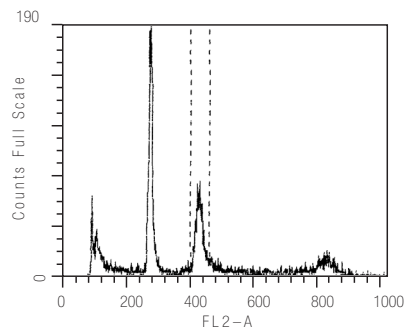


Figure 3b

Figure 3 DNA content analysis by flow cytometry of a testicular carcinoma stained with PI, showing populations with different DNA contents. (3a) Dot plot showing FL2-Width versus FL2-Area. (3b) DNA histogram, with some nuclei having a DNA index of 1.54. The DNA index was used as the basis for sorting.

Results

The DNA aneuploid peaks were sorted and stained using the Papanicolaou procedure for morphological examination or the Feulgen Azure A procedure for DNA measurement by light microscopy. Most samples showed acceptable morphology (Figure 1a) but some near-diploid sorted samples showed excessive autolysis (Figure 1b). The DNA index of sorted nuclei was confirmed by image analysis for several near-diploid and DNA-aneuploid samples. Autolysed nuclei and inflammatory cells were excluded based on morphological selection. Figure 2 shows the DNA index as determined by flow and image cytometry for these samples.

In samples classified as diploid ($n=27$), numerical chromosome aberrations were found in 15 (55.6%) samples. In populations with a DNA index >0.8 or <1.25 (near-diploid samples, $n=9$), three (33.3%) samples showed no aberrations with the probes tested. Numerical aberrations were found for all DNA aneuploid cells with a DNA index higher than 1.25 ($n=14$). Four of the 14 samples (28.6%) had flow or image histograms suggesting tetraploidy. These four samples had fluorescence in situ hybridization results suggesting the presence of four or more ($n=3$), or three ($n=1$), of the chromosomes being probed.

A sample showing multiple populations on the basis of DNA index (Figure 3) was analyzed by in situ hybridization. When stained for chromosome one, the population showed heterogeneity, with some nuclei having one, two, three, or four spots (Figure 4). The nuclei that had a DNA index of 1.54 were sorted and analyzed by in situ hybridization, and a large majority showed three spots (Figure 5).

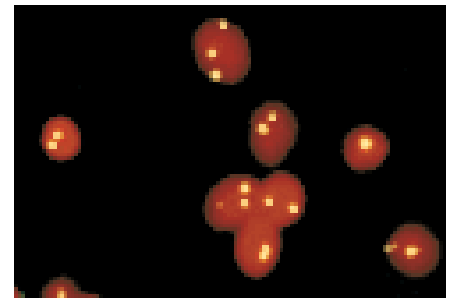


Figure 4 Detection of chromosome 1 in nuclei from a testicular carcinoma, with an α -satellite-specific, biotin-labeled probe. The unsorted population shown in the histogram in Figure 3 was cytospun and probed. The hybridization shows a mixed population of cells with one, two, three, and four spots.

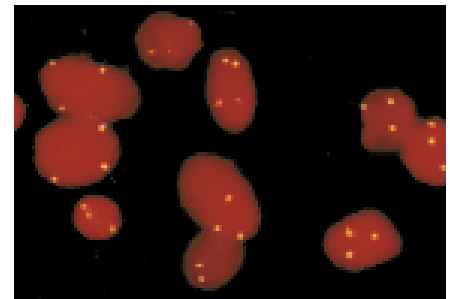


Figure 5 Detection of chromosome 1 in nuclei sorted from the mixed population shown in Figures 3 and 4 on the basis of their DNA index of 1.54. Three spots were observed in nearly all cells.

BD Biosciences publishes this

method as a service to investigators.

Detailed support for non-flow

cytometric aspects of this

procedure might not be

available from BD Biosciences.

Discussion

Sorting nuclei based on DNA index allows further analysis of populations that appear DNA aneuploid. One type of analysis is morphological assessment using light microscopy. This analysis has the potential for eliminating material giving false DNA ploidy peaks, such as autolytic⁵ or inflammatory cells. A second type of analysis, in situ hybridization, affords a means of confirming chromosomal aberrations, especially in potentially confusing cases of near diploidy. Finally, it is also possible to measure the DNA index using image processing on sorted nuclei.

Hints

While sorting of some samples in this study was performed with the flow rate set to MED, the coefficient of variation for DNA histograms is best when the LO setting is used. This might be important for samples in which the aneuploid population is very near diploid. Sorting performance is also best when using the LO flow rate.

References

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