**Background.** The objective of the current pilot project was to assess the efficacy of laser scanning cytometry (LSC) for DNA ploidy analysis of atypical urologic cytology specimens to enhance the distinction between benign and malignant changes.

**Methods.** Forty selected urologic cytology specimens that previously had been categorized as normal, atypical, or malignant were studied. Nuclear propidium iodide and fluorescence intensity measurements were converted to pixel values, which were used to create scattergrams that excluded debris and cell clusters from ploidy analysis, creating a gated (isolated) region of predominantly single cells for LSC ploidy analysis. Integral histograms then were created to show the number of cells present in diploid, tetraploid, and aneuploid peaks; these histograms also were used to assess DNA ploidy.

**Results.** Ten normal specimens, 10 malignant specimens, and 20 atypical specimens were examined to assess the efficacy of LSC ploidy analysis. Normal and malignant specimens generated reference histograms for comparison with the atypical specimens and exhibited 90% specificity and 100% sensitivity. Ten atypical aneuploid specimens had histogram and scattergram patterns similar to those produced by malignant specimens and, using the cytometer’s relocation feature, the presence of atypical cells was confirmed in the aneuploid regions.

**Conclusions.** The authors determined that DNA ploidy analysis of atypical urologic cytology specimens using LSC is a useful adjunct tool for identifying malignant specimens that lack sufficient cytologic criteria for diagnosis by light microscopy alone. However, LSC is time consuming and requires expensive equipment.

**Keywords:** DNA ploidy, laser scanning cytometry, urothelial carcinoma, urothelial atypia, cytology.

Urothelial carcinoma (UC) accounts for 90% of bladder carcinoma diagnoses. An initial diagnosis of UC usually is determined by cytologic evaluation of voided, catheterized, and/or ureteric urine specimens. Cytodiagnosis of high-grade tumors is sensitive and specific, but the diagnosis of low-grade tumors is more challenging and has relatively low sensitivity. Evaluation of DNA ploidy is one approach that is used to increase the sensitivity of UC detection. Genomic ploidy provides information about DNA content, with aneuploidy being a marker of neoplasia. Aneuploidy, tumor grade, tumor recurrence, and metastases have been shown by many studies to be strongly correlated. Two commonly used methods of ploidy measurement are flow cytometry (FC) and image analysis (IA).

FC assesses ploidy by measuring the fluorescence intensity of propidium iodide (PI)-stained cells as they flow past a laser beam.
is rapid, accurate, provides quantitative analysis, and is suited for automation. However, its disadvantages are the requirement of a large sample volume (from 10,00012 cells to 50,00013 cells) and the loss of cells after analysis. Furthermore, FC offers no method for visualizing subpopulations of cells within heterogeneous populations, or for examining cell morphology. Static IA of ploidy entails computerized measurement of cells stained with Feulgen stain.3 This approach requires few cells, and visual analysis of individual cells is possible. However, IA is slow and requires trained personnel to select cells manually.15,16 Sampling error usually is due to the small number of cells analyzed.

The laser scanning cytometer (CompuCyte Corp., Cambridge, MA) was developed to combine the elements of FC and IA9,10,17–22 and shows high ploidy concordance rates with respect to both of those methods.20,21 It is a microscope-based cytofluorometer that analyzes staining intensity of cells fixed to a glass slide. Cells are maintained after analysis, and the position of each cell on the slide is documented for future relocation. These features allow reanalysis of the same individual cells using another stain (Pap stain) or marker (antibody).

Laser scanning cytometry (LSC) has been used for ploidy analysis of routine cytologic specimens,10 touch preparations of urinary tract epithelial tumors,9 colorectal carcinomas,21 and head and neck squamous cell carcinomas.23 Immunocytochemical analysis of fine-needle aspiration specimens of breast carcinoma24 and melanoma metastases,25 immunophenotypic analysis of hematologic specimens,26 and cyclin expression in cytopsins of mechanically disrupted tumor tissue22 also have been performed using LSC. The current study was a prospective evaluation of 40 samples that was performed to determine the ability of LSC to evaluate aneuploid cells in atypical and malignant urologic specimens and to examine a possible role for LSC in identifying neoplasia in specimens reported as atypical.

**MATERIALS AND METHODS**

**Cytologic Specimens**

Forty selected urologic cytology specimens, including 14 voided urine samples, 19 barbotage urine samples, 1 catheterized urine sample, and 6 upper urinary tract brushings, were examined in the current study. Specimens were residual vial urine samples that were processed in the Department of Cytopathology at Fletcher Allen Health Care (Burlington, VT) and had been categorized as normal, atypical, or malignant based on cytopathologic assessment of Papanicolaou (Pap)-stained slides. Surgical specimens, when obtained, were used for the definitive final diagnosis. This study was performed according to guidelines approved by the Institutional Review Board of the University of Vermont (Burlington, VT).

**Slide Preparation**

One or 2 ThinPrep slides (Cytyc Corporation, Boxborough, MA) were prepared for each sample, depending on the volume of discarded material, producing a uniform layer of cells in a defined circular region (diameter, 2.9 cm) on each slide. Before staining, the slides were placed in ethanol and stored at room temperature. Slides were stained with propidium iodide (PI; 25 μg/mL; Sigma Chemical Company, St. Louis, MO) containing RNase A (200 μg/mL; Sigma Chemical Company) in phosphate buffered saline (PBS) for 30 minutes at room temperature in a dark, humidified chamber. PI stains both DNA and RNA; thus, RNase A was included to remove RNA. The slides were coverslipped using 1% n-propyl gallate in 50% glycerol:50% PBS and stored overnight in the dark at 4 °C to allow stabilization of the staining equilibrium.

**LSC Analysis**

Ploidy was assessed using the cytometer equipped with an Olympus BX-50 epifluorescence microscope (Olympus Optical Company, Tokyo, Japan) with a computer-controlled stage. Nuclear PI staining was excited with an argon ion laser (488 nm), and fluorescence intensity was measured using WinCyte acquisition and analysis software (CompuCyte Corp.) and converted into digital values (pixels). The total cell area on the slide was scanned automatically using a × 20 objective. The position of each nucleus (based on PI staining) was saved automatically as an xy coordinate address for future relocation.

Within each nucleus, the area or total number of pixels, the maximal pixel value, and the integral or sum of all pixel values were recorded. Maximal pixel values reflected the degree of chromatin condensation and allowed discrimination between G1/S (diffuse chromatin) and G2/M (condensed chromatin) phases of the cell cycle.15 Malignant cells and apoptotic cells also exhibited hyperchromicity and, thus, high maximal pixel values.19 Integral pixel values reflected total nuclear DNA content (ploidy).

These parameters were displayed as scattergrams, plots in which two variables are shown in relation to each other and in which each point represents an individual cell. Scattergrams of area versus maximal pixel value were used to exclude debris and cell clusters from ploidy analysis, creating a gated (isolated) region of predominantly single cells. Area versus integral scattergrams were produced from the gated re-
gion. Integral histograms were then created to show the number of cells present in diploid, tetraploid, and aneuploid peaks and were used for assessment of ploidy. Normal cell populations contain mostly diploid G0/G1 cells (DNA index /H11005 1.0) with smaller numbers of tetraploid G2/M cells (DNA index /H11005 2). Aneuploid populations contain abnormal numbers of cells with a DNA index /H11022 2.0 (hyperdiploid) or /H11022 2.0.

After ploidy analysis, PI stain was removed in PBS, and the slides were restained with Pap stain. Using the brightfield setting on the cytometer’s microscope, cell morphology then was assessed by relocating individual cells present in each of the histogram peaks, with special attention to cells in the aneuploid peak. Furthermore, using scattergrams of area versus maximal pixel value, relocation of events (nuclei) that exhibited large areas allowed the visualization of cell clusters.

RESULTS

Ten normal specimens and 10 malignant specimens were examined to assess the efficacy of LSC ploidy analysis. Normal G0, urothelial cells, squamous cells, and inflammatory cells were localized to the diploid peak (DNA index = 1.0), whereas G2/M urothelial cells and doublets of normal cells were present in the tetraploid peak (DNA index = 2.0) of the area versus integral histogram. Abnormal cells and clumps of normal cells comprised the aneuploid region (DNA index > 2.0) of the histogram. Normal and malignant specimens generated reference histograms for comparison with the atypical specimens and exhibited 90% specificity and 100% sensitivity.

The specimen type, cytologic diagnosis, ploidy status, and follow-up information for each specimen are shown in Table 1. Correlations between LSC ploidy results and histology results are shown in Table 2.
Specimens Diagnosed as Normal
On LSC analysis, all but 1 specimen that was diagnosed as normal by cytology exhibited a diploid histogram with 1 major diploid peak, 1 small tetraploid peak, and 0.2–2.2% (≤ 3%) of cells present in the aneuploid region (Fig. 1A). Upon relocation, the majority of cells present in the aneuploid region were small clumps of normal cells. One voided urine specimen (Table 1) produced an aneuploid histogram with an abnormally high number of cells (12.7%) present in the aneuploid region (DNA index > 2.0) (Fig. 1B). In that specimen, relocation identified multinucleated umbrella cells, not malignant urothelial cells, in the aneuploid region.

Specimens Diagnosed as Malignant
The malignant specimens that exhibited a distinct aneuploid peak on the histogram contained > 5% of the total cell population in the aneuploid region (Fig. 2A), as described previously. Specimens with 3–5% of the total cell population in the aneuploid region did not produce a clear peak on the histogram, but the presence of an aneuploid population was evident on the corresponding area versus integral scattergram. On relocation, visualization of individual events (nuclei) in this population revealed the presence of abnormal cells (Fig. 2B). Thus, data obtained from the scattergram were useful in specimens with low populations of malignant cells. Based on results obtained from the normal and malignant specimens, the presence of aneuploidy as detected by LSC was indicated when > 3% of the cell population was present in the aneuploid region of the histogram.

Six of the cytologically malignant specimens (Ta-

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**TABLE 2**
Correlation between Follow-Up Histologic Diagnosis and Laser Scanning Cytometric Ploidy Findings

<table>
<thead>
<tr>
<th>Follow-up histologic diagnosis</th>
<th>Total no. of patients</th>
<th>Ploidy as determined by LSC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Diploid</td>
</tr>
<tr>
<td>Specimens diagnosed as normal by cytology (n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No follow-up</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Specimens diagnosed as malignant by cytology (n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
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<td>0</td>
</tr>
<tr>
<td>Positive</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
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<tr>
<td>Specimens diagnosed as atypical by cytology (n = 20)</td>
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<tr>
<td>No follow-up</td>
<td>11</td>
<td>7</td>
</tr>
</tbody>
</table>

LSC: laser scanning cytometry.
* This voided specimen contained numerous multinucleated umbrella cells.
B This voided specimen contained few urothelial cells.

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**FIGURE 1.** (A) A representative Papanicolaou (Pap)-stained normal urothelial cell (DNA index = 1.02) with corresponding scattergram and histogram, both of which show a diploid pattern (arrow indicates cell location on both the scattergram and the histogram). (B) A representative Pap-stained multinucleated umbrella cell (DNA index = 6.02) with corresponding histogram showing an aneuploid population (arrow indicates cell location on the histogram).
Table 1) produced an aneuploid histogram peak that contained 6.0–36.3% of the cells (Fig. 2A). Three specimens demonstrated a small aneuploid population on the scattergram of only 3.1–3.3% total cells (Fig. 2B). In each specimen, the presence of malignant cells in the aneuploid region was verified by morphologic examination upon relocation. In one specimen (Table 1), a barbotage urine sample diagnosed as Grade 1 UC, the malignant cell population was localized between the diploid and tetraploid regions of the histogram and the scattergram (Fig. 2C). Although the malignant cells exhibited a normal area (nuclear size) on the scattergram, they possessed abnormally high maximum pixel values, suggesting a high degree of chromatin condensation, which is characteristic of malignancy. Relocation of the Pap-stained cells confirmed the presence of malignant cells in this population.

Five of the cytologically diagnosed tumors (Table 1) were UC in situ and exhibited similar histogram patterns with prominent aneuploid populations (Fig. 3A), demonstrating a strong correlation between aneuploidy and UC in situ. Two specimens (Table 1), both of which were Grade 2 UC, consisted of upper urinary tract brushings and exhibited similar histogram patterns, with tight aneuploid peaks at DNA index = 3.0 (Fig. 3B).

**Specimens Diagnosed as Atypical**

Of the 20 atypical specimens examined, LSC analysis found that 10 specimens exhibited an aneuploid popu-
Five of these specimens produced an aneuploid histogram (5.7–7.2% of cells) (see Fig. 2A): Four specimens exhibited aneuploidy only on the scattergram (3.0–4.6% of cells), as shown in Figure 2B; and 1 specimen showed an atypical cell population between the diploid and tetraploid regions of histogram and scattergram, as shown in Figure 2C. In eight specimens that exhibited aneuploidy, relocation of the aneuploid region verified the presence of atypical cells. In one specimen of voided urine with no follow-up information, relocation identified degenerating nuclei in the aneuploid region and atypical cells in clusters. The final specimen, a barbotage urine sample with no follow-up information, was similar to the malignant specimen (described above) that exhibited an aneuploid population localizing between the diploid and tetraploid regions. Relocation of these cells confirmed the presence of a small population of atypical urothelial cells.

Histologic follow-up was available for six of the atypical aneuploid specimens, and all six were positive for the presence of malignancy (Table 2). Despite the small atypical populations in some specimens, analysis of both histogram and scattergram revealed aneuploidy when >3% of total cells were in the aneuploid region. Subsequent relocation confirmed the presence of atypical cells with abnormal DNA ploidy in each specimen. Four specimens did not have biopsy follow-up, and further clinical surveillance will be required to determine the outcome of those patients.

Ten atypical specimens (Table 1) demonstrated a diploid histogram pattern (<3% of total cells in the aneuploid region, as determined from the normal and malignant specimens) with 0.0–2.6% of cells in the aneuploid region. In all 10 specimens, atypical cells were found in cell clusters, which were characterized by slight cellular pleomorphism. Because the LSC ploidy analysis was based on single cells (see Materials and Methods, above), these clusters were excluded, thus resulting in a normal diploid pattern. However, on the scattergrams of area versus maximal pixel value, atypical cell clusters were visualized as events with a large area. Four atypical diploid specimens of barbotage urine with no follow-up information exhibited overall low cellularity. Very few atypical cells were present on the slide, and although they were found in the aneuploid region, upon relocation for morphologic examination, <3% of all cells were in the aneuploid region. Thus, according to the criteria described above, these specimens also were considered diploid.

Follow-up data were not available for most of the atypical diploid specimens (7 of 10); however, 2 specimens were negative, and 1 specimen indicated the presence of a low-grade (Grade 1–2) papillary UC on histologic follow-up. The latter specimen demonstrated overall low cellularity with few urothelial cells. Definitive sensitivity and specificity values could not be assessed, because histologic and surgical follow-up data were not obtained on all atypical specimens.

**DISCUSSION**

In the current pilot project, we set out to assess the efficacy of using LSC in DNA ploidy analysis of atypical urologic cytology specimens to enhance the distinction between benign and malignant changes. The ThinPrep method of slide preparation was used, because it provides minimal cell overlap, maintenance of cell morphology, and limited cell loss between stainings. These were found to be important considerations, because slides were subjected to PI destaining and restaining by Pap stain so that individual cell morphology findings could be compared with ploidy results.

In a previous study of DNA ploidy using ThinPrep cytologic specimens and LSC analysis, Wojcik et al. demonstrated that whereas the ThinPrep method provided a direct, simple, and rapid method of staining, slides of low cellularity produced histograms that were difficult to interpret. The current study confirms this observation, underscoring the necessity of good-quality cytology preparations for accurate diagnosis. The results of the current study also suggest that LSC as-
essment should be performed only for specimens that have exhibited cellularity on prior evaluation, and preferably on specimens obtained by barbotage or brushing, rather than on routine voided specimens, which may be particularly low in cellularity.

The normal specimen that produced a ‘false’ aneuploid peak contained an abnormally high level of multinucleated umbrella cells. It has been reported previously\(^{16}\) that umbrella cells may contain aneuploid nuclei, which can lead to false ploidy results, especially with FC analysis. Although umbrella cells may be present at low levels in normal urine, they accounted for 12.7% total cells in this voided specimen. The cytometer’s relocation feature was critical in the identification of the unexpected aneuploid population. An excess exfoliation of surface umbrella cells may result from instrumentation, passage of renal stones, or assisted exfoliation (for example, brushing, particularly in ureteric specimens) but is unusual in voided specimens.

Kawamura et al.\(^{9}\) examined DNA ploidy by LSC analysis of tissue sections of urinary tract epithelial tumors after resection and found a strong correlation between aneuploidy and high-grade tumors; those authors also noted aneuploidy in specimens of UC in situ. Similarly, the current study of cytology specimens revealed aneuploid results in all specimens of high-grade UC and UC in situ.

The heterogeneous nature of urologic cytology specimens can affect LSC data. Cytology specimens of barbotage urine and upper urinary tract brushings consist of a high percentage of urothelial cells relative to populations of squamous cells and inflammatory cells. This more uniform population of urothelial cells is due to instrumentation that disrupts urothelial cells in the bladder, ureters, and renal pelvis during collection. The six malignant specimens (Table 1) that produced aneuploid histograms were barbotage urine specimens and upper urinary tract brushings. This finding suggests that aneuploid specimens will produce a more distinct aneuploid peak (> 5% of total cells) if the majority of cells are urothelial cells.

In contrast, voided urine samples tend to consist of a more heterogeneous cell population containing higher percentages of squamous cells and inflammatory cells. These cell types contribute to the diploid peak, thus reducing the relative percentage of aneuploid urothelial cells. In this situation, the advantages of LSC ploidy analysis are the ability to examine scattergrams for small populations of aneuploid cells and the ability to relocate to these areas for visual assessment of cell morphology. Identification of such small populations of abnormal cells may go undetected by other approaches, such as FC.\(^{15}\) The two malignant specimens that required scattergrams for ploidy analysis were voided urine samples that contained many squamous cells and inflammatory cells. Although urothelial cells were present, the overall percentage was too small to detect (3–5% of total cells) on the histogram, due to the high non-UC diploid cell population. Thus, whereas an aneuploid cell population may be present in these specimens, scattergram analysis may be required for ploidy determination.

LSC ploidy analysis provides increased sensitivity for the detection of small populations of abnormal cells compared with other approaches, such as FC.\(^{15,27–29}\) Validation of the presence of aberrant cells in the aneuploid peak is not possible in FC, leading to a variable number of false-positive aneuploid samples.\(^{28}\) Furthermore, compared with IA, LSC ploidy analysis can detect small abnormal cell populations within specimens of high cellularity. Although IA can detect small populations of abnormal cells (1–10%), the total number of cells examined is also small (150–200 cells).\(^{30}\) LSC ploidy analysis, therefore, allows the visualization of small subpopulations of abnormal cells within large populations of cells using the scattergrams and the cytometer’s relocation feature for morphologic confirmation.

The diagnosis of atypia covers a broad range in urologic cytology. This range includes specimens that contain few abnormal cells or cells with nuclear criteria that fall short of the criteria required for a diagnosis of malignancy. An atypical diagnosis also incorporates specimens that contain cohesive clusters, which may be the only cytologic evidence of papillary architecture. In voided specimens, an atypical diagnosis is assigned if atypical clusters of cells are present. Furthermore, atypia may include nonneoplastic morphologic changes due to virus, inflammation, instrumentation, chemotherapy, radiation, or bacillus Calmette–Guerin therapy.\(^{5}\)

Slaton et al.\(^{2}\) reported that the UC recurrence rate was higher in atypical cytologic specimens identified as aneuploid, with current or subsequent tumor recurrence in 20% of patients with atypical specimens of abnormal DNA ploidy, compared with 5% of patients with atypical specimens of normal DNA ploidy. This finding underscores the importance of detecting aneuploidy in atypical specimens. In the current study, ploidy aberrations were found in 50% of atypical specimens (Table 2). Atypical aneuploid specimens exhibited histogram and scattergram patterns similar to those produced by malignant specimens, and using the cytometer’s relocation feature, the presence of atypical cells was confirmed in the aneuploid regions. Although aneuploid cell populations among atypical specimens tend to be smaller than aneuploid cell pop-
ulations among malignant specimens, LSC ploidy analysis provided increased sensitivity in identifying abnormal cells in these atypical specimens.

In one atypical aneuploid specimen of voided urine with no follow-up information, false aneuploidy was produced by degenerating nuclei. PI intercalates into DNA; thus, PI uptake is greater when the DNA structure has opened up (degenerating nuclei).

Degenerating nuclei are most likely to be present in voided samples collected in the morning (after remaining in the bladder overnight). This finding may be a common occurrence that can be resolved by complementing LSC ploidy analysis with examination of cell morphology.

The 10 atypical but diploid specimens exhibited ploidy patterns similar to those produced by normal specimens. Relocation identified small numbers of atypical cells in the aneuploid region and in pleomorphic clusters, which were excluded from ploidy analysis because cell clusters were gated out in the analysis. Although the cytometer allows isolation and visualization of pleomorphic clusters, they are omitted from the analysis, and concomitant fluorescence in situ hybridization (FISH) analysis may be useful in the identification of genome abnormalities in these cell clusters.

In the current study, one atypical diploid specimen was shown on biopsy to contain low-grade (Grade 1–2) papillary UC. It should be kept in mind that low-grade UC lesions exhibit both diploid and aneuploid patterns, with ~80% of papillary urothelial neoplasms being diploid lesions. Diagnoses of this nature are unlikely to be made cytologically or by LSC, and final diagnoses will require surgical specimens.

Therefore, LSC can act as a confirmatory modality in high-grade lesions that are diagnosed by cytology as malignant. This may be useful when biopsies are not received or if the initial biopsy does not detect neoplasia (for example, in lesions of the ureters or in flat bladder lesions). However, LSC appears to make a more significant contribution in cytologic specimens that exhibit incomplete criteria for malignancy and are designated as atypical or suspicious. If LSC ploidy analysis using the cytometer’s relocation feature determines that these atypical cells are aneuploid, then a more confident diagnosis or prediction of neoplasia can be issued.

LSC ploidy analysis of atypical urologic cytology specimens can provide important additional information to routine cytology by detecting small populations of atypical urothelial cells. Factors that may produce artifactual results on FC, such as multinucleated cells, heterogeneous cell populations, variable specimen cellularity, atypical cell clusters, and cell degeneration, are resolved on LSC by visual examination.

In conclusion, we determined that DNA ploidy analysis of atypical urologic cytology specimens with LSC is a useful adjunctive tool for identifying those malignant tumors that lack sufficient cytologic criteria for diagnosis by light microscopy alone. However, the method is time consuming and requires expensive equipment. Another approach that allows the examination of individual cells on a slide and has been used successfully for the analysis of chromosome aneuploidy in cytologic specimens is interphase FISH. A multitarget, multiprobe, interphase FISH assay (UroVysion; Vysis, Downers Grove, IL) has demonstrated increased sensitivity compared with cytology alone in the detection of UC in urology specimens, and this assay is currently being introduced into clinical practice. A study examining LSC and FISH technology for the assessment of atypical urologic specimens currently is underway in our laboratory.

REFERENCES


14. Atkin N. Frequency of hyperdiploid chromosome complements in endometrioid tumors of the endometrium whereas similar tumors in the ovary tend to show hypodiploidy: a significant difference that may not be distinguishable by flow cytometry of DNA content. *Cytogenet Genome Res.* 2002;97:39–42.


33. Bubendorf L, Grilli B, Sauter G, Mihatsch M, Gasser T, Dalquen P. Multiprobe FISH for enhanced detection of similar tumors in the ovary tend to show hypodiploidy: a significant difference that may not be distinguishable by flow cytometry of DNA content. *Cytometry.* 1993;14:478–481.
