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DNA Quantification in Cervical Intraepithelial Neoplasia Thick Tissue Sections by Confocal Laser Scanning Microscopy

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Abstract Image analysis of tissue biopsies for determination of DNA content as an early marker of neoplasia is hampered by the complexity of corrections necessary to deal with nuclear truncation and overlap in thin sections. The use of confocal laser scanning microscopy (CLSM) for measurement of cellular DNA content on whole cells within thick tissue sections offers the advantage of preservation of cellular architecture, capacity for 3-dimensional analysis, and absence of sectioning artifacts. We have applied this technique to pararosaniline-Feulgen stained human cervical tissues graded from normal to cervical intraepithelial neoplasia (CIN) III. For the purpose of comparison, 15 μm sections were stained and mapped so that the same cell population could be analyzed by both integrated optical density and fluorescence intensity. Distribution of DNA content from normal cervical epithelial cells 2–3 layers out from the basal cell layer measured by both methodologies showed a stable G0/G1 population with no observable S-phase or G2 cells. Cells measured from areas of increasing CIN grade showed progressively higher DNA content values that were not observable in normal tissue. Although these data are preliminary they suggest that CLSM can be used to identify aneuploid states within defined structural areas of pre-invasive neoplasia. J. Cell. Biochem. 25S:49–56.

Key words: cervical intraepithelial neoplasia; confocal microscopy; DNA quantitation

INTRODUCTION

Alterations in cellular DNA content represent one expression of genomic instability which may drive accumulation of mutation, replication, amplification, or loss events, causing dysregulation of cellular homeostatic controls and predisposition to neoplasia.

As an early marker of genomic instability, the occurrence of aneuploidy has predictive value for progression and survival in a variety of tumor systems. Aneuploidy occurs with increasing frequency in colonic mucosa with increasing degree of atypia and appears to precede overexpression of mutant p53 [1]. It has independent prognostic value in less advanced stages of colon cancer but appears unrelated when tumors progress to Duke's stage C [2,3]. In transitional cell carcinoma of the bladder, aneuploidy is related to recurrence and disease-free survival [4,5] but not always as an independent predictive variable [6]. In lung, aneuploid DNA content can be more frequently observed in bronchial squamous metaplasia with increasing degree of atypia and is uniformly found in both early and advanced stage squamous cell carcinomas [7]. Patients with aneuploid non-small-cell carcinomas have significantly shorter survival than those with diploid tumors [8].

Although many studies of breast neoplasia support association between outcome and aneuploidy, this inference is clouded by the heterogeneity inherent in this disease process [9]. Disaggregation of breast biopsy samples hampers association of early pathologic features with genetic and DNA content abnormalities by mixing benign host and atypical cell populations associated with the primary tumor. That such an association in premalignant lesions should occur is supported by identification of cells having aneuploid DNA content arising from benign fibrocystic change [10], in macroscopically normal cells at distant sites from primary aneu-

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ploid but not diploid tumors [11], and within areas of atypical hyperplasia [12–15].

In addition to its usefulness as a measure of genomic instability involved early in the pathway of neoplastic progression, DNA content can be modulated in response to chemotherapy, providing a measure of residual target organ post-treatment instability. Cytophotometric or flow based determination of DNA content following the first course of combined cisplatin/5-fluorouracil treatment of head and neck squamous cell carcinoma demonstrated a shift toward euploidy and a decrease of the most highly aneuploid cell populations [16,17]. Those who failed to respond showed a persistence of aneuploid tumor cells. A similar response to combined cisplatin, methotrexate, and vinblastine chemotherapy could not be shown for invasive transitional cell carcinoma of the bladder [18].

Rigaut et al. [19] introduced the use of confocal laser scanning microscopy on thick section tissue blocks for quantitative measurement of DNA content while preserving the 3-D architecture within pathologically relevant zones. Although the coefficients of variation (CV) for control diploid and tetraploid rat hepatocyte peaks using standard intensity image acquisition parameters were larger than can be achieved by image cytometry (19% vs. 3–5%), DNA histograms were able to show differences between in situ carcinoma and surrounding normal epithelium. This observation lead us to investigate the use of photon counting capability in confocal microscopy to measure integrated fluorescence intensity achieved from pararosaniline Feulgen staining on the same cell population measured by optical density with static image cytometry. Since previous studies on cervical cytology specimens have demonstrated increasing relative optical densities from normal epithelium, through increasing grade of interepithelial neoplasia to invasive cancer [20,21], we chose cervical neoplasia as the model system for the present work.

MATERIALS AND METHODS

Sample Preparation

Archival paraffin blocks were obtained from the Department of Pathology , Medical College of Ohio, Toledo, Ohio. Embedded tissues had been routinely fixed in neutral buffered formalin. An initial 6 μm section was cut for histologic evaluation, serial 15 μm sections were then cut and mounted on aminopropyltriethoxysilane (Sigma Chemical Co, St. Louis, MO) coated slides. The sections were then baked at 57°C for 0.5 hr, deparaffinized in xylene (2 × 10 min) and rehydrated through graded concentrations of ethyl alcohol (100, 95, and 80%, 2 min each). An initial digestion with 0.25% trypsin in Hanks' balanced salts (Life Technologies, Grand Island, NY) was carried out for 2 h at 37°C to remove partial cells from the top surface of the section and simplify the image analysis. Longer digestion times tended to cause separation of the epithelium at the basal cell layer and some movement of tissue across the slide surface or complete loss of tissue. Decondensation of chromatin as described by Hyytinen et al. [22] was accomplished by heating at 90°C for 3 min in 50% glycerol. The Feulgen staining reaction was then carried out essentially as described by Allison et al. [23]. Briefly, 5N HCl with polyethylene glycol (15g PEG/40 ml HCl) was used for hydrolysis for 1 h at 25°C followed by one rinse in Schiff reagent (Sigma) and staining for 45 min. Excess reagent was removed by 3 successive washes in SO_2-water (1g sodium metabisulfite/200 ml H_2O:100 ml 1 N HCl). Slides were then dehydrated through graded alcohols to xylene and mounted using immersion oil (N_D = 1.515).

Image Mapping and Analysis

With laser power reduced to 1% and a ×20 objective in line, the confocal microscope was used to generate a wide field map of each area of interest as defined by the pathologist. Cells were then first located and measured by integrated optical density (IOD) using a CAS 200S (Becton Dickinson, San Jose, CA) off the absorption peak at a wavelength of 540 nm with a ×100 objective. The normal human cellular DNA content of 7.18 pg was used with the mean epithelial cell optical density for histologically normal cervix and the mean stromal cell optical density for CIN lesions to arrive at a conversion factor for DNA content per cell. As pararosaniline dye gives strong fluorescence at 630 nm, these same cells could be measured by confocal microscopy. Confocal images (Bio-Rad MRC 600) for analysis were acquired using a high numerical aperture (Nikon 60X Plan Apochromat, NA 1.4) objective and 1.65 electronic zoom factor to give 6 pixels per micron by accumulating 6 scans per 1 μm optical section. The design principles of confocal microscopy operate to restrict out of focus light from reaching detection by a photomultiplier tube, allowing optical sections of 1 μm or less to be collected and thus provide

exceptional spatial resolution. The confocal aperture used here is optimal for the ×60 objective. Linearity of response, photobleaching and attenuation of fluorescence emission by depth of tissue section are important obstacles to overcome. We made use of the capacity of the Bio-Rad MRC-600 system for discrete counting of photon emission events as the laser scans each pixel in the image field. Gain was fixed at a value of 8.0 and black level adjusted to insure that less than 10 emission events per pixel per scan (the linear capacity of the instrument) were detectable. Three-dimensional volumes were reconstructed using VoxelView 2.1.2 (Vital Images, Inc., Fairfield, IA). By setting a limiting threshold of voxel value for detection, the detection algorithm operates from a chosen seed location within the cell of interest to identify all neighboring voxels within the cellular volume. Detected voxel values are summed to give a measure of total intensity. Any mapped cells found to lie at either the upper or lower boundary surface from 3-D reconstruction were eliminated from analysis by both procedures. Preservation of cellular architecture, allowing DNA quantification on specific cells within defined areas of interest, is perhaps the major advantage of working with tissue sections. If conventional 6 μm sections are used, however, the need for algorithms attempting to correct IOD for nuclear truncation artifacts is introduced. While significant progress in this area is being made, these correction algorithms may prove to be tissue specific, substantially increasing the time required for their development [24]. Edge detection capability of image cytometers is also severely challenged by cellular overlap routinely found within tissue sections and particularly within areas of neoplasia characterized by proliferative activity. If section thickness is increased to 15 μm, 2–3 layers of whole cells can be visualized by Feulgen staining eliminating assumptions necessary in dealing with nuclear truncation. The architecture of normal cervical epithelium is advantageous as a model system in that cells 2–3 layers away from the basal layer are well spaced in x, y, and z planes with stable diploid DNA content, differentiating gradually in size to the squamous border.

RESULTS

For comparison of IOD and integrated fluorescence procedures on cells sampled from the same mapped population, a single patient block was chosen which contained areas of normal and early stage cervical lesions (CIN I and II). Measured cells are numbered in the top half of each figure. As shown in Figure 1, DNA content measured on a CAS 200S microdensitometer is characterized by a well defined diploid peak (CV = 9%) showing no S-phase or G2 cells. Initially, IOD was measured on a test population of cells, screened to eliminate those at the top or bottom surface, to establish a lower IOD threshold. This threshold was subsequently used to filter truncated cells from analysis. Parameters characterizing the distribution of DNA values around the 2C peaks are given in Table I. Sample variances calculated on square root transformed data were significantly different. A limited number of whole cells could similarly be identified within areas corresponding to lower grade cervical lesions (CIN I–II) from the same tissue section (Fig. 2A,B), although with greater difficulty. In this manner, four cell populations were identified by conventional cytophotometry and mapped for subsequent measurement by integrated fluorescence intensity from CLSM. The extent of nuclear overlap from a lesion of high grade (CIN III, Fig. 3) obtained from a separate patient block precluded analysis by IOD. Although some mapped cells could not be reliably measured by both techniques, it is readily apparent that most cells which could be separated for analysis by CLSM have DNA contents far outside the range seen for normal cervical epithelium.

DISCUSSION

Quantification of Cellular Fluorescence Using Confocal Laser Scanning Microscopy

Previous work in quantitative assessment of multidrug resistance and DNA content in neurons from the human cerebellum or rat hepatocytes by CLSM have identified potential utility and problems associated with fluorescence measurements [19,25,26]. As discussed by Rigaut et al. [19], the effects of attenuation and bleaching can be corrected by fitting parameters to the nonlinear curve defined by image intensity vs. tissue depth. This is particularly relevant to work with very thick tissue sections (50–100 μm) where multiple scans (typically 10–15) per

TABLE I. 2C Peak Distribution Parameters for CAS 200S vs. Confocal Measurement

Method	Mean	Variance	Skewness
CAS 200S	7.26	.43	.96
Confocal	7.17	1.03	.30

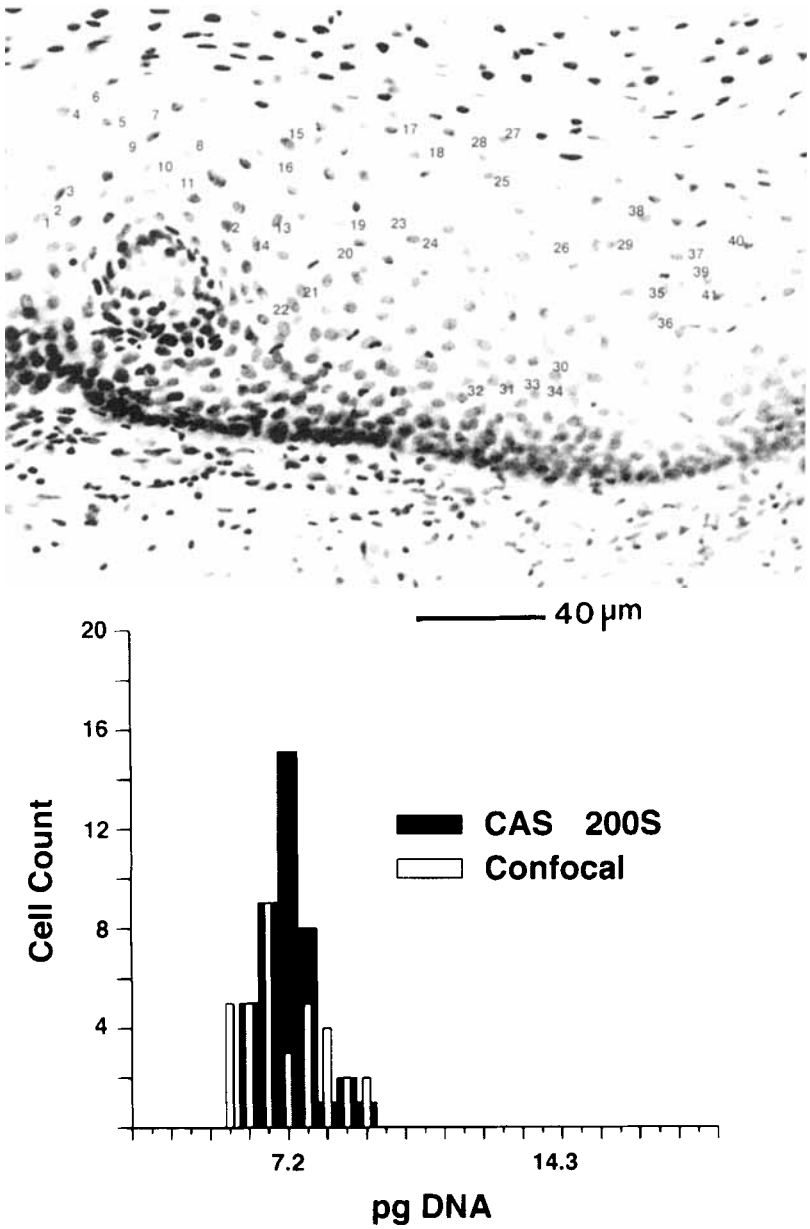


Fig. 1 Distribution of DNA content in pararosaniline-Feulgen stained cells of histologically normal cervical epithelium. Numbered cells identify the population used for measurement of DNA content both by optical density and fluorescence intensity (original magnification = ×200, scale bar = 40 μm).

field are averaged since bleaching is not restricted to the plane of focus but continuous throughout the entire area exposed to the laser. Bleaching and attenuation effects were judged to represent a minor component of experimental error in the present studies with 15 μm thick sections when 6 scans per focal plane were averaged. Cells vary in their susceptibility to acid hydrolysis due to chromatin condensation and linearity of fluorescence response falls off with increasing dye incorporation [27,28]. Tissue used for analysis here was heated in 50% glycerol to promote decondensation [22] and lightly stained while still allowing optical density measurements. Linearity of fluorescence intensity with incorporation of pararosaniline dye is likely to be the dominant error component, affecting quantification of fluorescence intensity to a greater extent than optical density.

Alterations of DNA Content With Increasing Grade in Cervical Intraepithelial Neoplasia

Areas of normal epithelium and lower grade CIN I–II lesions were located within the same

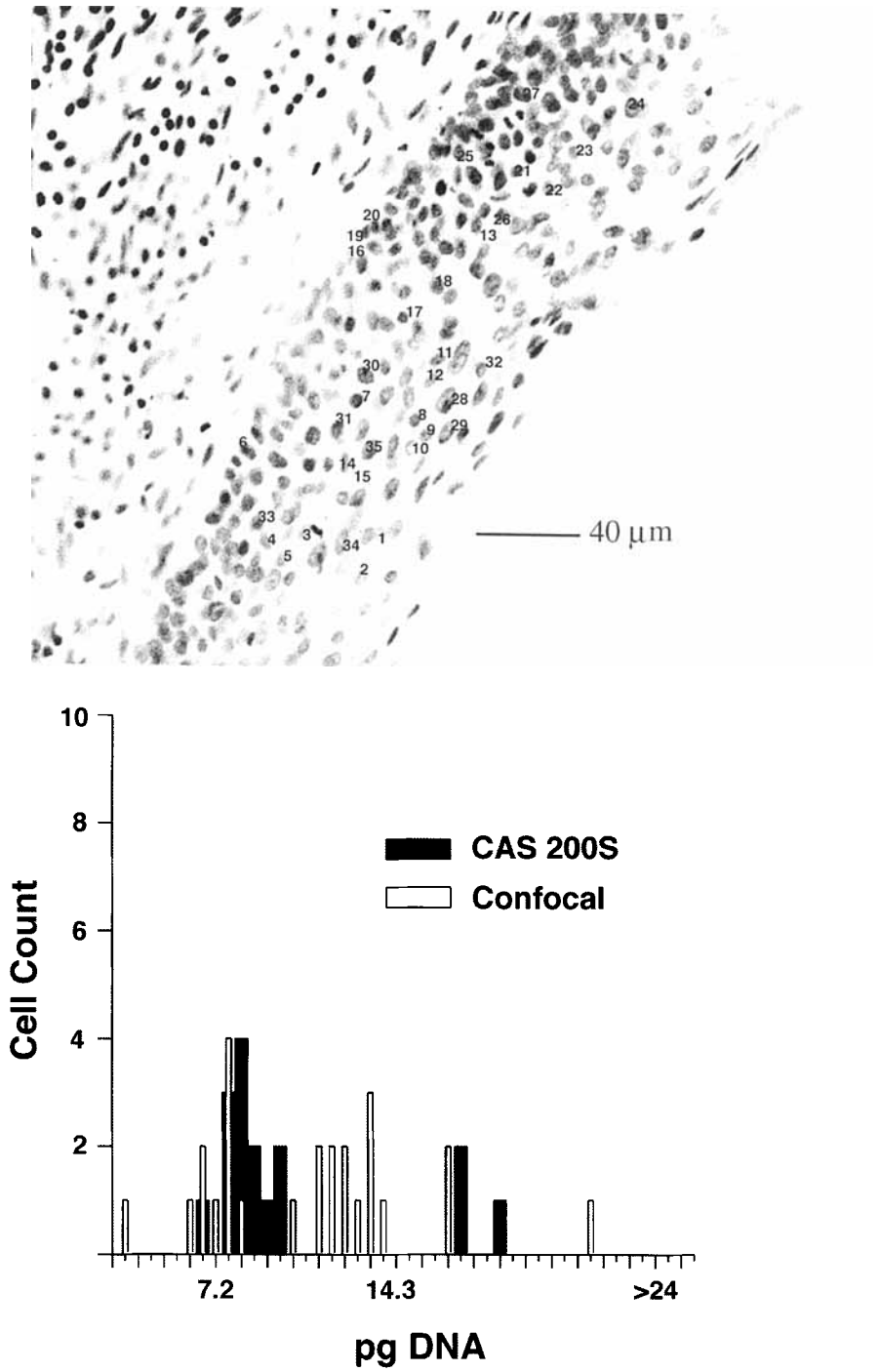
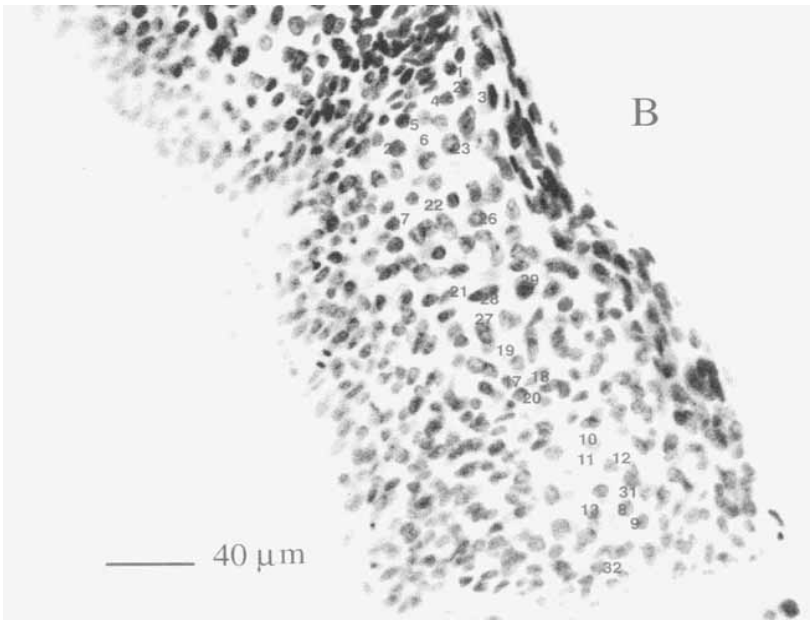


Fig. 2. A,B: Distribution of DNA content in pararosaniline-Feulgen stained cells of low grade. Areas chosen for analysis are from the same tissue section described for Figure 1. Numbered cells identify the population used for measurement of DNA content both by optical density and fluorescence intensity(original magnification = ×200, scale bar = 40 µm).

tissue section. In Figure 1 it is apparent that the distribution of DNA content determined by CLSM matches closely with that determined by IOD and indicates a stable G0/G1 cell population. Cells included for analysis in Figure 2A were chosen from an area of low grade. Although relatively few cells could be identified by IOD, results show DNA content values shifted to the right that are totally absent from normal epithelium. This pattern is more pronounced in Figure 2B from an area of higher grade. Here the distribution is substantially



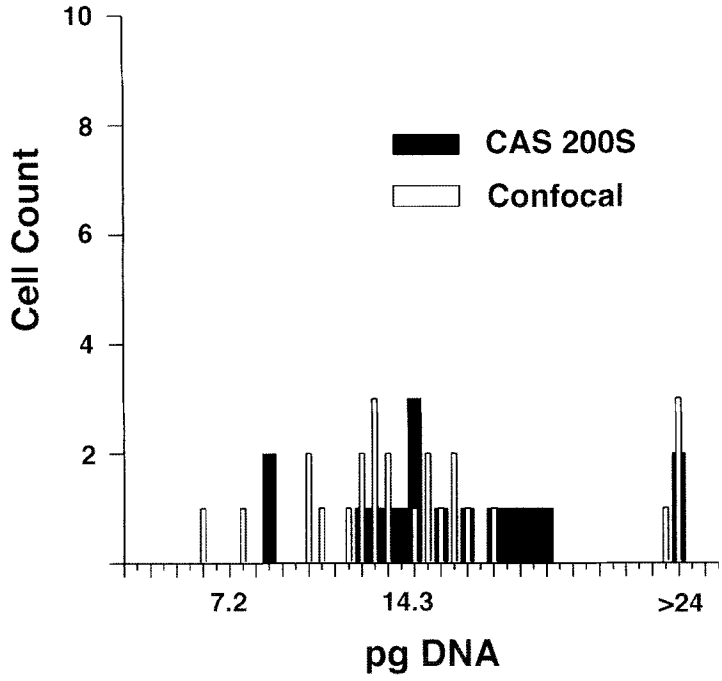
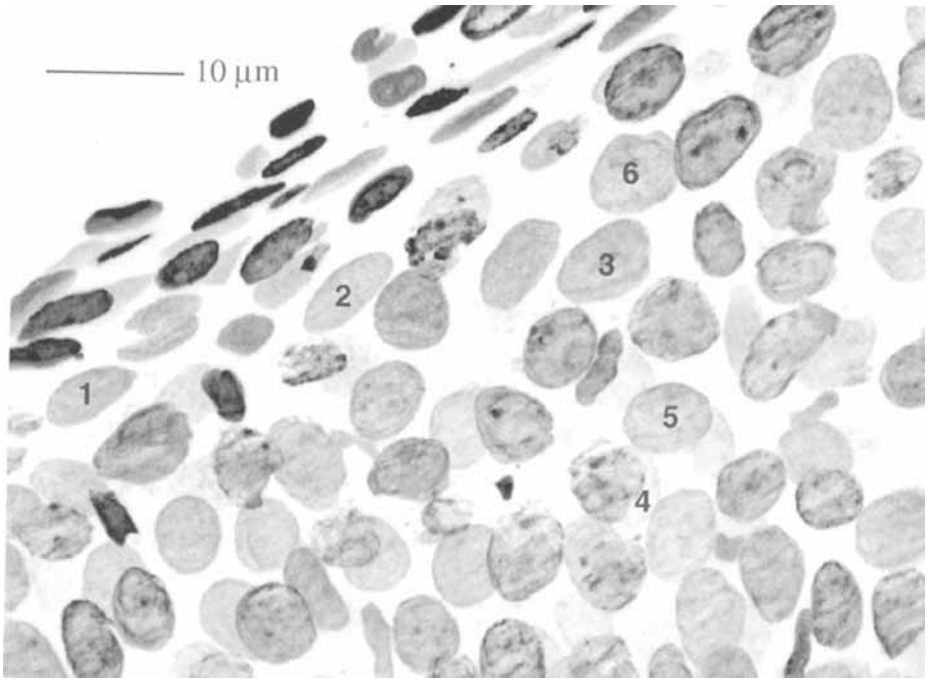


Figure 2. (Continued.)

shifted to the right with some cells beyond 6c. While these results are still preliminary, they demonstrate in a model system 1) that similar DNA distributions can be obtained by both measurement methodologies; 2) that DNA content increases progressively with increasing grade in cervical interepithelial neoplasia in agreement with previous studies [20,29]; and 3) suggest that the resolving power of confocal microscopy can enable discrete measurement on cells separated by 1 μm in depth (the z-step size used throughout) making possible the analysis of more complex histologic features than possible for density based imaging methodologies.

SUMMARY

Whole cells within 15 μm thick sections of cervical epithelium showing normal epithelium or graded levels of CIN were analyzed by both microdensitometry and CLSM. Analysis of



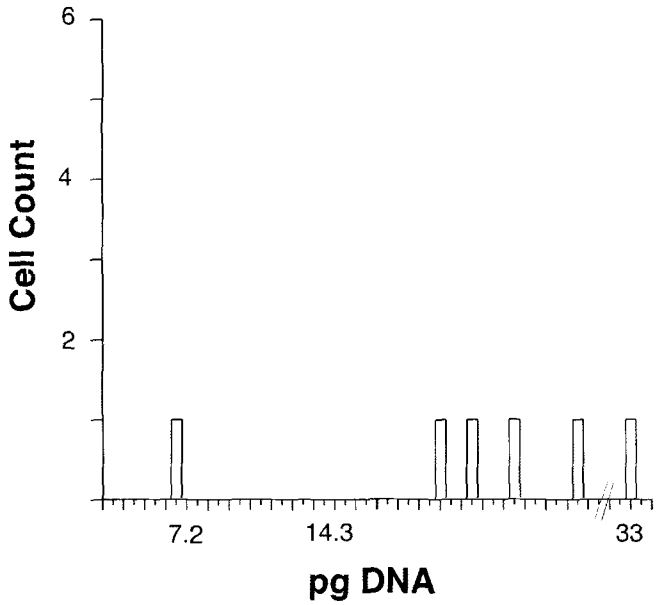


Fig. 3. Distribution of DNA content in pararosaniline-Feulgen stained cells of high grade. Selected cells could only be isolated for measurement by CLSM (original magnification = ×600, scale bar = 20 μm).

whole cells precludes the necessity of correction algorithms required for thin sections. The greater resolving power of the confocal microscope allows isolation and measurement of individual cells that cannot be accomplished by conventional transmitted light densitometry. Although the distribution of DNA content is greater than can be achieved by IOD on isolated dispersed cells, CIN is associated with the presence of cells greater in size and DNA content than observed in normal cervical epithelium. These results suggest that CLSM can be of advantage in analysis of thick tissue sections (>15 μm) at early stages of neoplasia to identify

alterations in DNA content as a measure of genomic instability. This is not possible by conventional densitometry.

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