10 Diagnostic DNA Cytometry of the Urothelium

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CONTENTS

10.1 Introduction

The subjective cytologic examination of urinary sediment by no means offers an ideal solution to the diagnosis of bladder carcinoma:

- The *sensitivity* is only 70%, depending on the grade of tumor malignancy.
- The *specificity* is only 80%–95% (Murphy et al. 1986; Rübben et al. 1989).
- The *reproducibility* of malignancy grading, at approximately 60%–70%, is poor (Ooms et al. 1989).
- The *prognostic relevance* of the cytologic tumor grade is not adequate to have a definite impact on treatment planning (Böcking et al. 1990).
- The *prospective biologic behavior* of dysplastic cells cannot be accurately predicted.
- The *labor cost* is high for the screening of large sample volumes in subjects at risk.

Since the 1960s, the latest *cytometric techniques* have been applied in an effort to furnish objective, reproducible data on urothelial cells, and thus help solve the problems listed above (Tavares et al. 1966; Koss et al. 1975; Foss and Kaalhus 1976a,b; Tribukait and Esposti 1978). The earliest results, and still the most promising to date, have been supplied by *DNA cytometry*, which was formerly done using microphotometers and today employs TV image analysis systems or flow cytometers.

Tumor cytogenetics forms the biological basis for *diagnostic DNA cytometry*, which involves the detection of aneuploid chromosome sets in neoplasia and relates the variability of this aneuploidy to the grade of tumor malignancy. Attempts have also been made to use TV image analysis (morphometry) of the urothelial cells to find solutions to the foregoing problems. Here we shall discuss the scientific results of these investigations in terms of their *relevance to routine cytologic diagnosis* and its clinical application.

10.2 Morphometry of the Urothelium

Koss et al. (1975), unlike Fossa and Kaalhus (1976a), were unable to find a significant difference in nuclear sizes between normal and malignant urothelial cells. In a sophisticated image-analysis study of 117 bladder carcinomas, these authors found no significant correlation between various nuclear parameters (size and its variability, chromatin density and distribution) and clinical tumor stage or length of patient survival. Aikens and Liedtke (1982) used a high-resolution microscopic cytophotometric system in an attempt to discriminate between normal and malignant urothelial cells. The results were ultimately disappointing. Even more recent studies with TV image analysis

systems (Montironi et al. 1985) can at best demonstrate significant differences between the large groups of all benign and malignant urothelial changes but cannot furnish results relevant to the diagnosis of individual cases. Consequently, morphometric image analysis is no longer being seriously considered as a tool for the evaluation of problem cases or the grading of malignancy in urinary cytology. At the same time, image-analysis parameters can be successfully employed in automated systems when the goal is to replace the screening function of a human assistant by a machine, rather than to enhance the confidence and prognostic relevance of a cytologic diagnosis already made by an examiner (see Sect. 10.7).

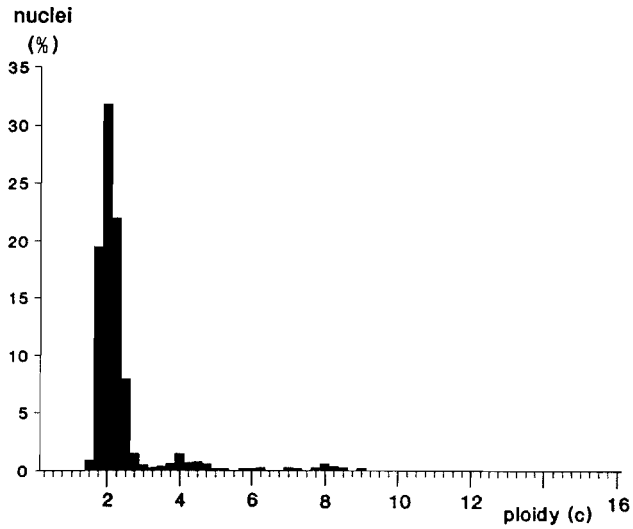

Fig. 10.1. **Summation histogram of DNA distribution in 5000 normal urothelial cells from urine samples of 50 different subjects.** For each subject 100 Feulgen-stained cells were measured using the MIAMED system (Wild-Leitz, Wetzlar); granulocytes served as reference cells (× 1.20)

10.3 Biologic Principles of DNA Cytometry

10.3.1 Discrimination

Except for gametes, the nuclei of human cells each contain two sets of 23 chromosomes (=2c). Four sets of chromosomes (=4c) are present in the G2 phase of the cell cycle prior to cell division. A multiple of the chromosome set corresponding to whole-number powers of the 2c value (i.e., 4c, 8c, 16c, 32c) regularly occurs as a physiologic phenomenon in some tissues. This process is called *euploid polyploidization*. Polyploid chromosome sets are consistently found, for example, in thyroid epithelium, seminal vesicle epithelium, hepatic epithelium, mesothelial cells, cardiac muscle fibers, and also in urothelial cells. As early as 1959, Walker demonstrated this in cytogenetic studies of the mouse urothelium. Several authors have used DNA cytometry to detect polyploidization of up to 8c in human urothelial cells (Levi et al. 1969; Fossa 1975; Farsund and Hostmark 1983). We detected 4c and 8c ploidies in 45 of 50 normal urothelial populations (Biesterfeld et al. 1992). Figure 10.1 shows a DNA summation histogram of these normal urothelial cell populations from spontaneously voided urine samples. Some cytologically detectable viral infections, such as HPV, can likewise induce a polyploid nuclear DNA content (Chatelain et al. 1989a), although this has not yet been demonstrated for the urothelium. In the synthesis phase of the cell cycle, a limited percentage of the cells (<10%) may show DNA contents that are between the integral powers of the 2c value (Sandritter 1981).

Aneuploidy refers to numerical or structural deviations from these normal chromosome sets and corresponding DNA contents (Fig. 10.2). Heim and Mittelman (1987) describe a 5p isochromosome, monosomy X, and trisomy 7 as primary chromosomal abnormalities associated with carcinoma of the urinary bladder. Pauwels et al. (1987), on the other hand, describe a loss of chromosome 5 and the deletion of a fragment from chromosome 19 as characteristic anomalies in urothelial neoplasms. A tumor that exhibits this aneuploidy can be identified as originating from the urothelium. This characteristic chromosomal aneuploidy is not readily detectable by DNA cytometry, however, because it corresponds to a loss of only about 2.5% of the nuclear DNA, or 0.05c. This is why *low-grade urothelial tumors are usually not identified as "aneuploid" by DNA cytometry, even though they are cytogenetically aneuploid.* This applies to almost all tumors, and there are virtually no "diploid" urothelial tumors. But cytometry is sometimes incapable of demonstrating chromosomal aneuploidies, leading many cytometrists to speak incorrectly of "diploid" tumors. Structural and numerical aberrations of chromosomes 1, 7, 11, 17, and others as well as marker chromosomes may appear as secondary abnormalities during the course of tumor progression, but they are not characteristic of urothelial tumors. Once these secondary chromosomal anomalies have appeared,

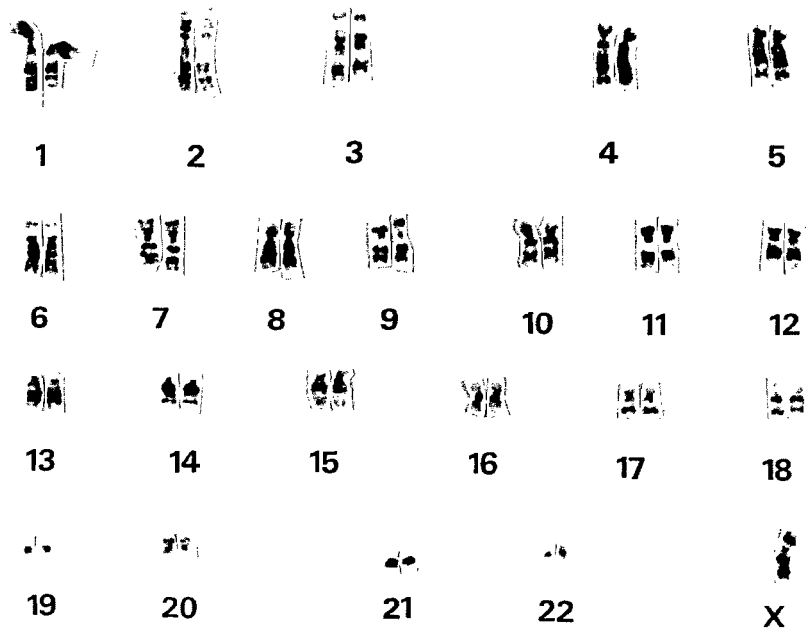

Fig. 10.2. **Karyotype of a representative tumor cell from a human grade 1 urothelial carcinoma.** Monosomy X, translocation of a fragment from chromosome 12 to 1, trisomy 20. (Specimen courtesy of Dr. Ott, Dr. Offner, Institute for Pathology, RWTH Aachen, FRG)

the nuclear DNA content can be successfully identified as abnormal or "aneuploid" even by cytophotometric analysis.

Chromosomal aneuploidy is viewed in cytogenetics as a *hard marker* for the neoplastic transformation of cells (Heim and Mittelman 1987). This means that the detection of cells with an aneuploid chromosome set is equivalent to the detection of neoplastically transformed cells (Atkin 1971; Huber 1986; Pauwels et al. 1987; Smeets 1987). For example, the cytogenetic detection of aneuploid cells is used as a marker for malignancy in the cytologic diagnosis of pleural effusions (Heim and Mittelman 1987). Some benign cells also may exhibit aneuploidy as a result of exposure to ionizing radiation or cytostatic agents. If the single-cell interpretation of DNA cytometric values is to be useful for detecting aneuploidy, these effects must be excluded.

Due to the *high cost of chromosomal preparations*, attention has shifted in the past 40 years from cytogenetic analysis to DNA cytometry as a means of evaluating cells for aneuploidy to detect neoplastic transformation (Atkin 1971; Sandritter and Böhm 1975; Ploem-Zaaijer et al. 1979; Barlogie et al. 1980; Wied et al. 1983; Hedley et al. 1984; Barlogie 1981). After classic Feulgen staining (Feulgen and Rossenbeck 1924), in which DNA is combined with a violet dye (parafuchsin) in a stoichiometrically precise fashion, the DNA content can be determined with reasonable accuracy by microdensitometric measurement of the dye content per nucleus and calibration with nuclei having a known, normal 2c content (reference cells).

Although aneuploidy is a chromosomal phenomenon of the individual cell, classic DNA cytophotometry relates this term to the most frequently occurring (modal) value in a cell population (the highest peak in the *DNA histogram*). In the case of a tumor, this value corresponds to the *"stemline"* (Seidel and Sandritter 1963). A single-cell interpretation of aneuploidy can be accomplished by DNA cytometry only in non-polyploidizing tissues (Böcking et al. 1984; Böcking 1990). If the modal value or tumor stemline is located in an "aneuploid region" of the DNA histogram (outside 2c, 4c, 8c, 16c), aneuploidy of the entire population is assumed, and the tumor is said to be "aneuploid" even if most and not all of the nuclei have an aneuploid DNA content. In polyploidizing tissues such as the urothelium, we classify a modal value outside of 2c, 4c, 8c, 16c ± 2c, 4c, 8c or 16c × CV as "aneuploid." Because CV is the coefficient of variation in the reference cell population (e.g., lymphocytes), less variation in the reference cell population means a better chance for the successful DNA cytometric detection of aneuploidy. This conservative DNA cytometric interpretation of aneuploidy is *very specific* but *relatively insensitive*, because aneuploidy is assumed to be present only if the majority of tumor cells display a quantitatively significant alteration in their chromosome sets. Generally this is the case only if the *quantity of DNA is increased by more than 10%*, corresponding to a modal value greater than 2.2c. In practical terms, this means that an urothelial carcinoma can be diagnosed by

the detection of an aneuploid stemline only if, beyond the primary chromosomal mutations, there have also been significant secondary mutations leading to a DNA increase >2.2c in the majority of the tumor cells.

10.3.2 Grading of Malignancy

It is known from cytogenetics that, in a number of solid carcinomas in humans, the degree of chromosomal aberrations correlates with the prognosis (Heim and Mittelman 1987).

Sandberg showed in 1986 that noninvasive bladder carcinomas of histologic grades 1 and 2 usually had normal diploid karyotypes and only occasionally exhibited marker chromosomes, whereas most grade 3 tumors were strongly aneuploid and contained abundant marker chromosomes. Superficially invasive grade 2 tumors displayed more aberrations than noninvasive tumors. The presence of *marker chromosomes* appears to be an absolute prerequisite for *invasive tumor growth* in bladder cancer patients (Summers et al. 1989). Massive chromosomal abnormalities were found mainly in aggressive tumors. In patients with noninvasive papillary tumors, recurrence developed in 90% of cases with marker chromosomes but in only 5% of cases with no chromosomal anomalies.

The *malignant potential of urothelial carcinomas* depends less on the most frequently occurring chromosomal anomaly (modal anomaly), as is commonly believed, than on the *variability ("range") of the secondary anomalies*. This results from the genetic instability of the tumor. The more the secondary chromosome anomalies vary from cell to cell in a urothelial carcinoma, the more malignant and aggressive the tumor (Pauwels et al. 1988). Modal chromosome counts <49 occurred in 97% of all grade 1 tumors but in only 49% of grade 2 and 8% of grade 3 tumors. While all infiltrating bladder carcinomas showed cells with more than 49 chromosomes, this was noted in only 22% of the noninfiltrating tumors (Pauwels et al. 1988). The chromosomal range of aneuploidies in DNA cytometry corresponds to the variance about the normal 2c value (=2c deviation index, 2cDI; Böcking et al. 1984). The higher the measured values and the greater their variability, the greater will be the variance about the 2c value. The loss of a stemline is considered a particularly unfavorable prognostic sign, as it also leads to a high variance of DNA values and to very high individual values. Variance is too abstract a prognostic index for the clinician, however, and it is more useful to work with a logarithmic conversion of the 2c deviation index (2cDI) in which the *DNA malignancy grade* (DNA MG) is assigned a value on a 0–3 scale. In this conversion the lowest possible variance of 0 represents a DNA malignancy grade of 0, while the highest observed value of 51 (for osteosarcoma) corresponds to a DNA malignancy grade of 3.0 (Böcking and Auffermann 1986). Follow-up studies to date have confirmed the *prognostic relevance* of this DNA malignancy grade for malignant lymphomas and for carcinomas of the prostate, breast, and bladder (Böcking et al. 1985, 1986a,b, 1988, 1989, 1990; Auffermann et al. 1986) (Figs. 10.3–10.5).

The above observations from tumor cytogenetics are consistent with observations of a statistically significant prognostic improvement in patients with bladder carcinomas that display a DNA stemline in the "diploid" or "tetraploid" range, as opposed to those with aneuploid stemlines in the 3c–6c range (Tavares et al. 1973, 1986).

At the same time, a review of the literature shows little consistency in the prognostically relevant interpretation of the DNA distribution in statistical and flow-cytometric DNA analyses. Frequently the authors confine their efforts to the subjective description of DNA histograms. Many nonstandard terms are employed in an effort to describe the characteristics of the DNA distribution, although most authors focus their attention on the position of the stemline. This refers to the most frequent (modal) value that is accompanied by a doubling peak corresponding to cells in the G2/M phase (Sandritter and Carl 1966). Authors have attempted to characterize the modal value with terms such as diploid, triploid, tetraploid, hyperpentaploid, near-hexaploid, hyperoctoploid, etc., without defining precisely what these terms mean. Other authors have described various prognostically relevant types of histogram in which the DNA distribution must be subjectively evaluated (Auer et al. 1980). Pfitzer et al. (1976) were among the first to emphasize the poor reproducibility of the subjective interpretation of histograms. Besides the 2c deviation index mentioned above, a variety of other indices have been devised for the more precise and objective interpretation of DNA histograms:

- The DNA index of the modal value (Barlogie et al. 1980)
- The mean nuclear DNA content (Sprenger et al. 1974)

- The diploid deviation quotient: DDQ (Fossa and Kaalhus 1977), which also is an expression of mean ploidy
- The percentage of nuclei with a DNA content >5c (Ploem-Zaaijer et al. 1979)
- The Z value (Sprenger et al. 1974), which expresses the ratio of "euploid" to "aneuploid" DNA values

The most widely used, prognostically relevant *differentiation into "diploid" and "aneuploid" tumors has no biological, cytogenetic basis*, because virtually all tumors are aneuploid at the chromosomal level. Since DNA cytometry reveals any number of transitional states between slight and profound deviations of the stemline modal value from the normal 2c value, an arbitrary division into "diploid" and "aneuploid" tumors makes little sense. It would be more useful to establish empirically derived, prognostically relevant thresholds of the modal value for specific tumor entities.

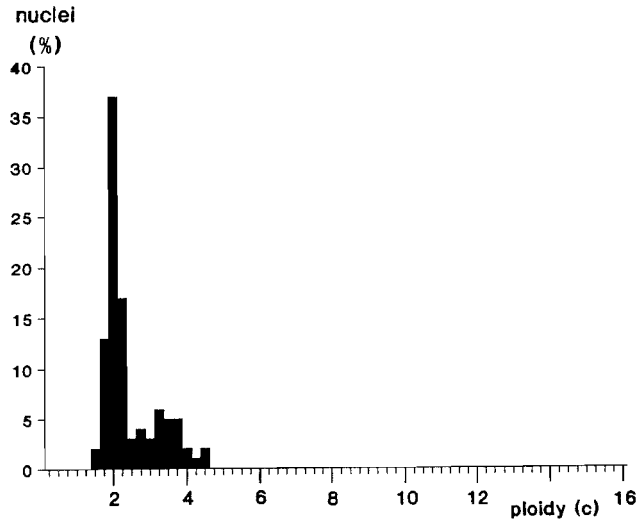

Fig. 10.3. **DNA histogram of a grade 1 urothelial carcinoma,** DNA malignancy grade 0.35

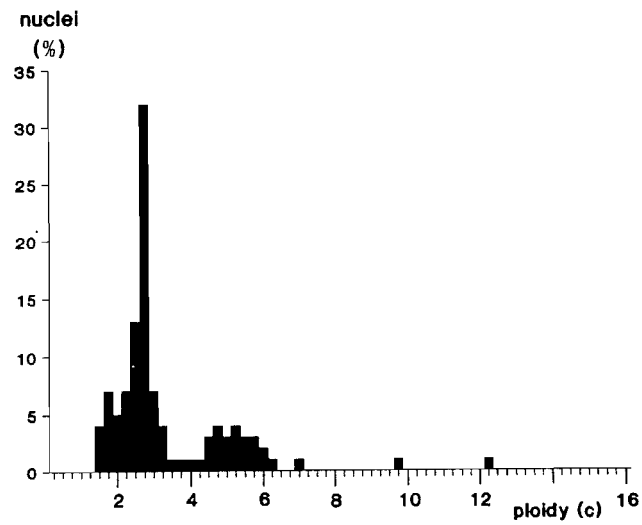

Fig. 10.4. **DNA histogram of a grade 2 urothelial carcinoma,** DNA malignancy grade 1.28

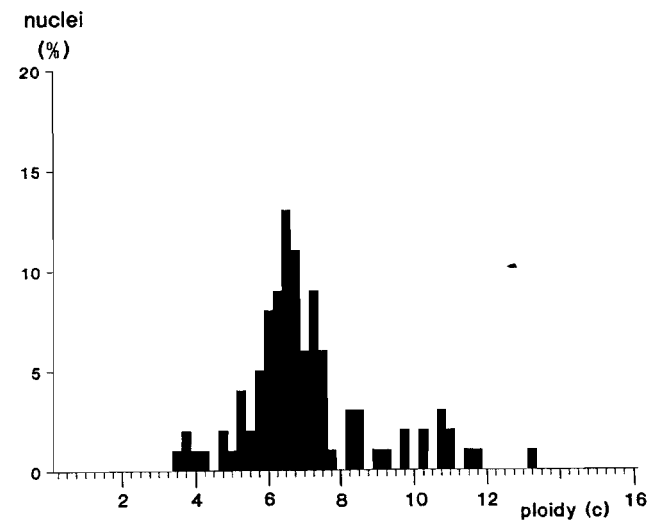

Fig. 10.5. **DNA histogram of a grade 3 urothelial carcinoma,** DNA malignancy grade 2.60

10.4 Specimen Processing

10.4.1 DNA Single-Cell Cytometry

DNA single-cell cytometry can be performed on routine cytologic material that has been processed by any standard cytopreparatory technique. However, the cells in the voided urine or washings should be fixed by the addition of 50% alcohol immediately after collection. Also, the cellular material should be concentrated in a monolayer to facilitate individual cell detection. Cytocentrifuge specimens are particularly suitable, but sediment smears are also acceptable.

Feulgen stain, necessary for *DNA cytometry* in visible light, can be applied to *any specimen regardless of its prior fixation or staining*. It is necessary only to (post)fix the specimen for at least 2 h in 4% formaldehyde in phosphate buffer at pH 7.0 prior to hydrolysis in hydrochloric acid. If cytologic specimens can be immediately fixed by this technique, postfixation is not required. Pure alcohol fixation adversely affects the proportionality of hydrolysis between the different cell types (Böhm et al. 1968). The former practice of adding acetic acid is not advised, as it leads to an increased scatter of measured values (Roels 1990). Besides forming free aldehyde groups on purine bases, the hydrochloric acid hydrolysis serves to destain previously stained cells. Coverslipped specimens

also can be used, as several hours' soaking in xylene will remove the coverslip while leaving the cells adherent to the slide. DNA cytometry can even be performed on tissue material fixed with formalin and embedded in paraffin, since there is a relatively simple technique for recovering individual cells from the embedded tissue (Delgado et al. 1984). Precise, stoichiometric, automated Feulgen staining can be performed overnight with a temperature-controlled staining machine (Chatelain et al. 1989b).

The use of fluorescent stains for interactive DNA single-cell cytometry is not recommended, because the fading effect makes it impossible to repeat the measurements on the same nuclei. This is occasionally necessary, though, for reasons of quality control. Cytometry in UV light also must be performed in a darkened room, requiring prolonged adaptation of the eyes.

Thus, a *special preparatory technique is not required* for DNA single-cell cytometry since any specimen, regardless of its previous fixation or staining, can be processed by applying appropriate postfixation and destaining procedures. The standard procedure for Feulgen staining is outlined in Table 10.1.

Table 10.1. **Steps in the Feulgen staining procedure**

No.	Time (min)	Contents of the cuvet	Function
1	60	4 % Formaldehyde in Sörensen buffer, pH 7.0	Fixation
2	5	Tap water, flowing	Rinse
3	2	Distilled water	Rinse
4	55	4 *N* HCl, 27.5 °C	Hydrolysis
5	5	Tap water, flowing	Stop hydrolysis
6	2	Distilled water	Rinse
7	60	Schiff's reagent[a]	Stain
8	10	SO_2 water[a]	Wash out excess dye
9	10	SO_2 water	Wash out excess dye
10	10	SO_2 water	Wash out excess dye
11	5	Tap water, flowing	Rinse
12	2	Distilled water	Rinse
13	5	70 % ethanol	Dehydration
14	5	96 % ethanol	Dehydration
15	5	100 % ethanol	Dehydration
16	5	Xylene	Clearing

[a] Prepared by method of Graumann (1953).

10.4.2 DNA Flow Cytometry

Cell samples for flow cytometric analysis are processed differently than samples for conventional urinary cytology.

Following immediate centrifugation (1500 rpm for 5 min), the sediment can be resuspended in 3 ml of phosphate buffer and either stained at once or fixed prior to staining. Several hours' fixation of the sediment in 95% alcohol is recommended to prevent autolysis and standardize the results of the measurement. Fluorescent staining of the resuspended sediment is performed overnight by adding 5 ml of propidium iodide or DAPI. The addition of a detergent (e.g., Triton X-100) destroys the cells while leaving their nuclei largely intact (Ratliff et al. 1985). Before cytometry, the cell suspension is sieved through a 40–70 µm nylon mesh filter. Deparaffinated tissue samples originally fixed in formalin can be analyzed by DNA flow cytometry following combined enzymatic-mechanical cell retrieval (Coon et al. 1986).

10.5 DNA Single-Cell Cytometry

10.5.1 Measurement Systems

Until a few years ago, the DNA content of individual cells was usually measured with *scanning microphotometers* (Deeley 1955) using photomultiplier tubes. Either the object is moved beneath the measuring beam (e.g., UMSP, Zeiss, Oberkochen, FRG), or a moving mirror is used to scan the beam across the object (integrated microdensitometer, e.g. Vickers M 86, Vickers, York, U.K.). The main advantage of these systems is their high gray-scale resolution; their main disadvantage is the relatively slow and complicated measurement process. Each individual nucleus must be manually positioned within the aperture of a mask. The subjective and thus imprecise determination of the individual background (blank value) of the nuclei represents a further limitation of this method (Auffermann et al. 1984). About 3–4 h is needed for the measurement of approximately 20 reference cells and 150 "diagnostic" cells. These instruments are poorly suited for routine diagnostic use, therefore.

Since the 1980s, scanning cytophotometers have increasingly been replaced by *TV image analysis systems* that employ a video camera connected to a microscope (Bachmann and Hinrichsen 1979;

Auffermann et al. 1984; Böcking et al. 1987) (Fig. 10.6). The image from the TV camera is processed by an image analysis computer, whose functions include the densitometric measurement of individual cells (Fig. 10.7). With this type of system, even nuclei that are closely adjacent or in contact with one another can be concurrently measured and their morphometric parameters determined. An individual background (blank) measurement can be performed for each nucleus. This method is so rapid and convenient that 20 reference cells and approximately 250 diagnostic cells can be measured in about 20 min. Potential disadvantages of nuclear DNA measurement with TV image analysis systems are shading (nonuniform image illumination and camera sensitivity) and glaring (scattered light in the microscope beam path). Both effects, however, can be corrected to a degree by appropriate hardware and software selection.

Unlike flow cytometric systems, *TV image analysis systems can perform measurements on routine cytologic and histologic specimens.* This eliminates the need for separate or repeat specimen collection with further processing. Feulgen staining is essential for DNA measurements, however. A suitable individual- and tissue-specific reference cell population can be selected for each specimen (e.g., squamous epithelial cells in the urine). Since each individual cell can be identified and classified prior to measurement, the relevant cells can be selectively evaluated without the concomitant measurement of artifacts. This also allows for "rare event detection," meaning the isolation and measurement of rare cells that are of special diagnostic interest. Thus, dysplastic cells in a bladder irrigation specimen containing various cell populations can be measured as selectively for benign/malignant discrimination as tumor cells can for the grading of malignancy. In this way various cell populations can be measured concurrently within the same sample. The immediate assignment of quantitative results to specific cells on the monitor provides for an immediate feedback of measured values (see Fig. 10.7). The ability to check results by relocating individual cells and remeasuring them offers a decisive advantage in terms of quality control. These systems also permit the determination of morphometric parameters such as nuclear shape, size (area), chromatin pattern and derivative parameters as well as the quantification of immunohistochemical reactions.

Although the number of measurable cells is somewhat decreased compared with flow cytometry, rapid advances in image analysis hardware have made it possible to measure 300 cells, including references cells, within a 20-min period

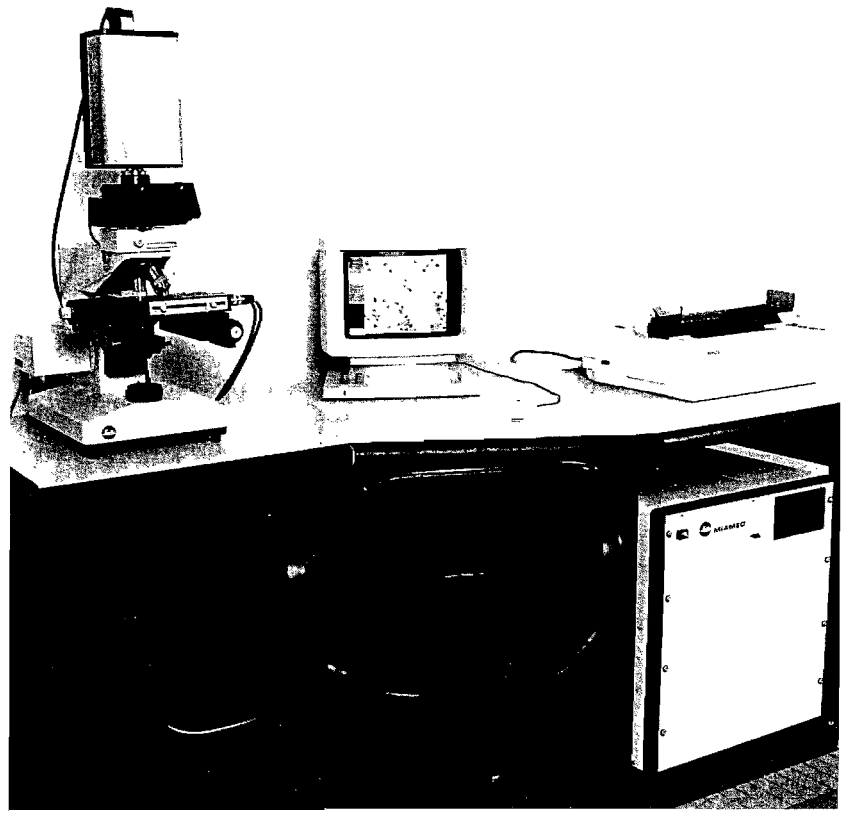

Fig. 10.6. **Interactive TV image analysis system** includes an automatic microscope for semiautomated diagnostic DNA measurements and the determination of nuclear morphometric parameters (MIAMED, Wild-Leitz, Wetzlar, FRG)

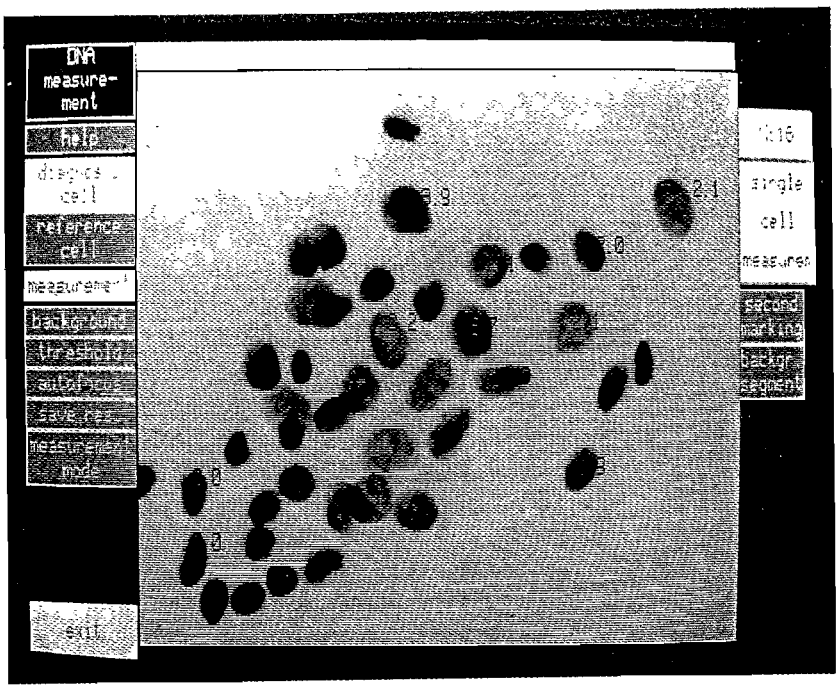

Fig. 10.7. **Interactive monitor of the MIAMED TV image analysis system** with screen-edge sensor fields and display of measured tumor-cell nuclei and their DNA contents in c (Feulgen stain)

(Böcking et al. 1992). Measurements of larger cell numbers do not significantly alter the observed DNA distribution (Marschner 1992). Interactive DNA image cytometry is particularly well suited for DNA measurements in urinary cytologic specimens, because it can be performed on existing smears or cytocentrifuge specimens that have already been evaluated by a cytologist. There is no need for special sample processing aside from Feulgen staining prior to the analysis. Relevant (dysplastic or tumor) cells can be measured within the normally diverse cell populations. After completion of the measurement, the results can be printed out in the form of a report sheet showing the DNA histogram and diagnostic analysis (Fig. 10.8).

10.5.2 Diagnostic Results

As stated in the section on the Biologic Principles of DNA Cytometry, this method is excellent for the identification of neoplastically transformed cells, for determining the benignancy or malignancy of cytologically or histologically diagnosed dysplasias (or borderline lesions), and for the grading of malignancies.

10.5.2.1 Identification of Neoplastic Urothelial Cells

Bass et al. (1989) found that the *sensitivity* of interactive DNA single-cell cytometry for the detection of malignant cells in urine samples was 88%, compared to only 58% with conventional Papanicolaou cytology (n=33). Even with grade 1 and 2 urothelial carcinoma, the sensitivity was still 86% (vs. 33% with cytology). These authors use the detection of cells >5c as markers for malignancy. The sensitivity of DNA cytometry for the identification of aneuploid cells in urothelial carcinoma, as in other tumors, is dependent on the grade of tumor malignancy. The lower the grade, the better the chance for the successful cytometric detection of aneuploidy.

The *specificity* of fluorescent image analysis was 96.7% in a study of 523 high-risk asymptomatic subjects (Parry and Hemstreet 1988). The rate of 3.3% false-positive diagnoses probably results from the approximately 8% prevalence of polyploid cells with >5c DNA content occurring normally in the urine (Biesterfeld et al. 1992). If the physiologic polyploidization of urothelial cells were taken into account in the interpretation of DNA measurements, the rate of false-positive diagnosis could probably be reduced. Koss et al. (1987) achieved a sensitivity of 60% with DNA single-cell cytometry in 30 tumor cell-positive urine samples versus only 23.2% with conventional Papanicolaou cytology. Because these authors

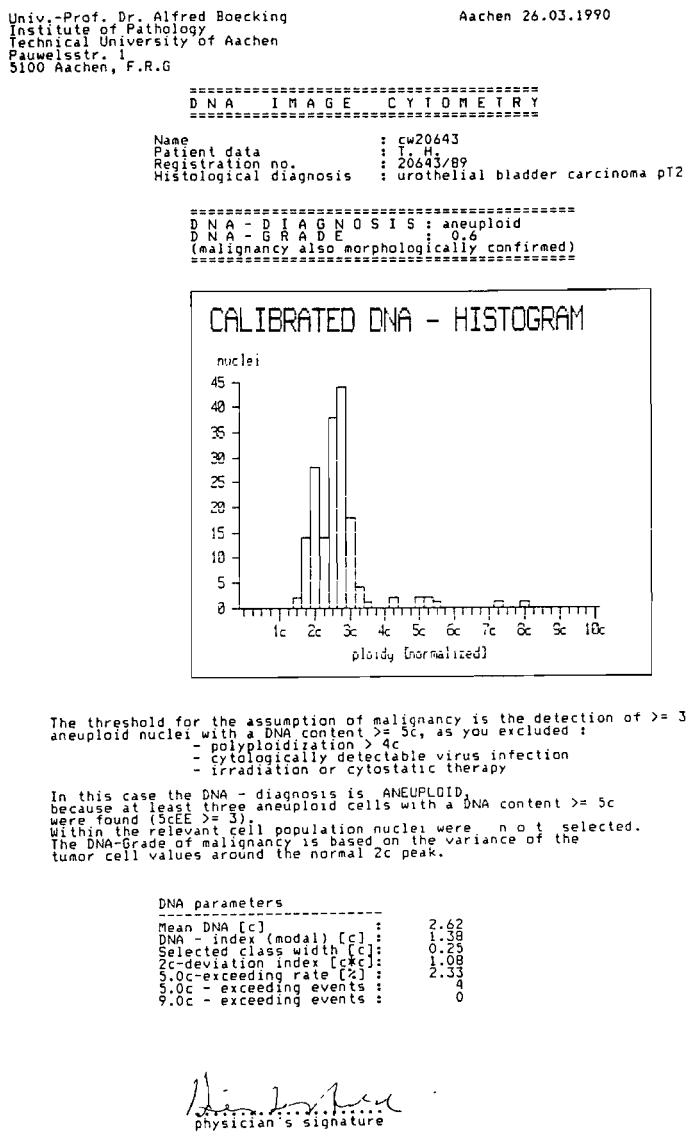

Fig. 10.8. **DNA cytometric printout for a grade 1 urothelial carcinoma, including the DNA histogram and diagnostic analysis:** aneuploid, DNA malignancy grade 0.6

used only the position of the stemline to detect aneuploidy ("diploid"/aneuploid) and did not also take into account individual aneuploid cells >5c, their sensitivity was significantly below that of Parry and Hemstreet (1988). Presumably the sensitivity and specificity of interactive DNA cytometry could be further improved by combining the single-cell and stemline detection of DNA aneuploidy and by taking into account the polyploidization of urothelial cells. We suggest that the identification of more than 10% cells >5c and/or a stemline outside the ranges of 2c, 4c, or 8c ± the standard deviation of the reference cell population should serve as the criterion for the DNA cytometric detection of aneuploidy, and thus of neoplasia, in urine samples. If one or both of the aneuploidy indices are positive, neoplasia may be assumed. Applied in this way, interactive DNA cytometry could increase the sensitivity of conventional urinary cytology.

10.5.2.2 Discriminating Benign and Malignant Dysplasias

Fossa (1977) found DNA aneuploidies suggestive of malignancy (cells >5c) in six of 12 moderate urothelial dysplasias in proximity to overt bladder carcinomas. In 50% of cases, then, malignancy could be diagnosed in the stage of dysplasia by the use of DNA cytometry. This suggests that dysplasias which exhibit an aneuploid DNA distribution (by single-cell or stemline interpretation) are prospectively malignant. We do not subscribe to the hypothesis that specific grades of dysplasia correlate with specific DNA distribution patterns, as there is no biological basis for this assumption. Rather, a dysplasia in which aneuploid cells are detected must be classified as prospectively neoplastic even though subjective morphology does not yet support that diagnosis. This is understandable when one considers that malignant transformation occurs at the DNA level and usually has chromosomal effects, whereas morphologic alterations appear later as epiphenomena. To date, however, there have been no precise studies on the prospective diagnosis and follow-up of malignancy in dysplastic urothelium.

10.5.2.3 Grading of Malignancy

Lederer et al. (1972) and Fossa (1975) were the first to document the suitability of the DNA distribution pattern as a grading parameter for bladder carcinoma. These authors used the position of the DNA stemline as their principal grading parameter but did not correlate their results with follow-up. Fossa et al. (1977) noted a statistically significant prolongation of survival in 63 patients with a "diploid" tumor stemline, compared with 60 patients with a "nondiploid" line. Hofstädter et al. (1984), in a study of 64 patients, found a close correlation of the stemline quotient SQ (equivalent to the modal DNA index) and diploid deviation quotient (DDQ; Fossa 1975) with the histologic grade according to Bergkvist et al. (1965) and the depth of tumor infiltration. The survival time of patients with "diploid" tumors (SQ =1.1) differed significantly from that of patients with aneuploid tumors

(SQ>1.1). It was found that the DDQ could be used to differentiate 30 groups of bladder cancer patients who had significantly different survival times. The frequency of tumor recurrence and the interval to recurrence also correlated significantly with the DDQ. Fossa and Kaalhus (1985) showed that the stemline quotient in 123 patients was prognostically relevant, regardless of the tumor stage. This means that the DNA determination had significant prognostic implications in every stage.

Böcking et al. (1990), in a study of 117 bladder cancer patients, investigated the prognostic influence of tumor stage (TNM), histologic grade (Mostofi et al. 1973), the subjective DNA histogram classification according to Fossa (1975), the mean DNA content, the differentiation of DNA stemlines into "diploid" versus aneuploid, and the DNA grade of malignancy (Böcking and Auffermann 1986). The TNM stage demonstrated the highest correlation with patient survival (Fig. 10.9). Multivariant regression analyses (Cox 1972) showed that histologic grade, another independent variable, demonstrated the second highest correlation with survival time (Fig. 10.10). When this variable was omitted from the models due to its insufficient interindividual reproducibility of only 62%, the DNA malignancy grade was introduced into the model as a further independent prognostic variable. Through DNA malignancy grading, it was possible to distinguish three patient groups with significantly different survival times (Fig. 10.11). The subjective histogram classification showed as little prognostic significance as the simple differentiation between "diploid" and aneuploid tumors. Nuclear size and its variability discriminated only two groups with significantly different lengths of survival. The interindividual reproducibility of DNA malignancy grading was investigated for 20 different tumors. With a correlation coefficient of r=0.97, it was significantly higher than that of subjective malignancy determinations, for which Ooms et al. (1983), for example, report a reproducibility of only 41%–58%. Thus, DNA malignancy grading provides a reproducible parameter that is equivalent to subjective morphologic grading in its prognostic relevance.

10.6 DNA Flow Cytometry

10.6.1 Measurement Systems

In DNA flow cytometry, isolated cells stained with a fluorescent dye that binds specifically to DNA (e.g., acridine orange, ethidium bromide, DAPI) flow in single file through a laser beam. The cells, enclosed by a fluid jet and accelerated under high pressure to a velocity of about 10 m/s, can be measured at a rate of at least 100 cells/s (e.g., FACStar plus, Becton-Dickinson, USA; Fig. 10.12). The minimum cell count in a measurable sample is 10 000, however, so hypocellular samples cannot be processed. The DNA content per cell corresponds to the level of emitted fluorescent light, which is measured by photomultipliers. The results are printed out as "scatter plots" and histograms and represent a summation result for the cell population as a whole, with no differentiation of specific subpopulations. *Advantages* of flow cytometry are that the results are available quickly (e.g., within 15 min) and are highly representative owing to the large number of measured cells. This results in a more precise determination of the DNA index of the tumor stemline. Another advantage is the ability to evaluate several parameters at once in the same sample, such as the nuclear DNA content, nuclear size, cellular protein content and, recently, various immunologic markers. This type of study utilizes several different labels in the same cell, which are measured at different wavelengths. Sophisticated systems (see Fig. 10.12) include a device for sorting out cells with specific detected properties, such as a DNA content >5c, for separate morphometric analysis in a TV image analysis system (Tanke et al. 1983). Flow cytometry also has several *disadvantages* compared with single-cell cytometry:

- The samples must contain *no fewer* than approximately 10 000 cells. Hypocellular samples must be analyzed by single-cell cytometry.
- Besides the routine cytologic specimens that are already available, an *additional sample* must be collected specifically for flow cytometry. To flow-process material from solid tumors, special cell recovery techniques must be used that employ mechanical and enzymatic measures (Vindelov et al. 1983; Hedley et al. 1983).
- Due to the lower resolution limit of approximately 1%, (tumor) cells that occur in the urine with a lower prevalence are not detected. Thus, *rare event detection is not possible*, and the sensi-

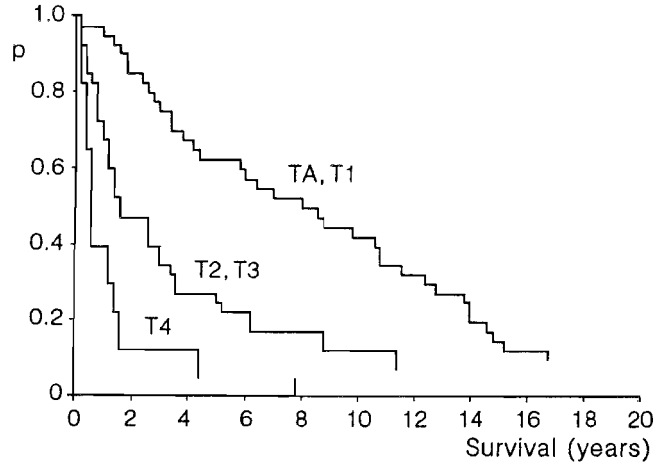

Fig. 10.9. **Kaplan-Meier survival curves for 117 patients with bladder carcinoma,** arranged according to pathologic stages pT1–4. (From Böcking et al. 1990)

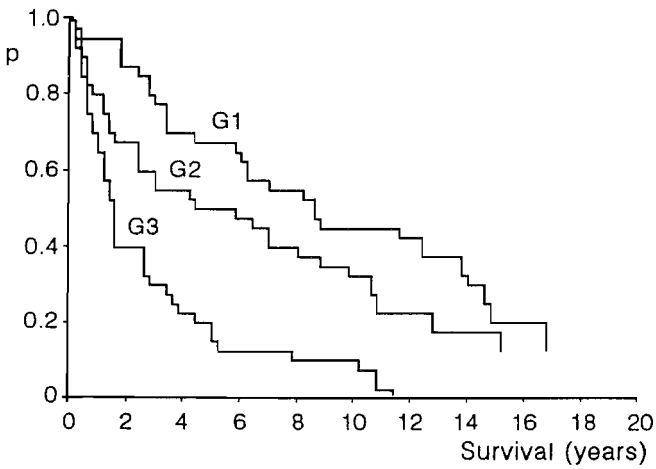

Fig. 10.10. **Kaplan-Meier survival curves for 117 patients with bladder carcinoma as a function of the histologic malignancy grade according to Mostofi et al.** (1973). (From Böcking et al. 1990)

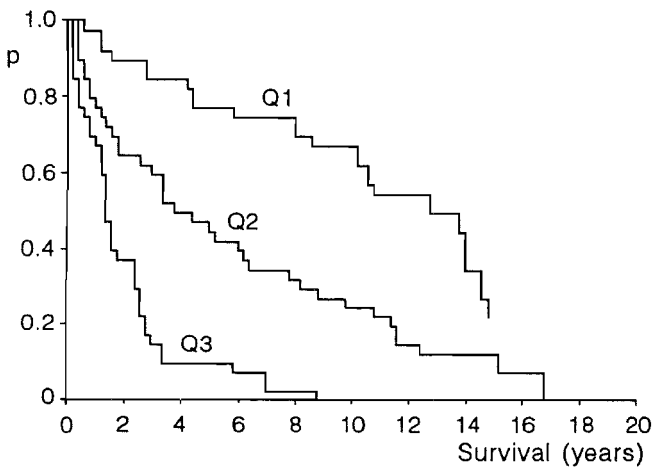

Fig. 10.11. **Kaplan-Meier survival curves for 117 patients with bladder carcinoma as a function of DNA malignancy grade.** (From Böcking et al. 1990)

tivity of the method is particularly limited by the low prevalence of atypical cells in low-grade and low-stage urothelial tumors.

- Because normal urothelial cells, squamous epithelial cells, inflammatory cells, and histiocytes are always measured in addition to questionable tumor cells and cannot be reliably differentiated from them, one cannot confidently assign the measurements to specific cell populations. Thus, DNA malignancy grading is not feasible in a mixed cell population. Because individual measured DNA values are not processed, there is no automatic accounting for smaller but prognostically relevant DNA values. Thus, DNA flow cytometry usually permits only a *rough* and biologically unwarranted *differentiation into "diploid" and "nondiploid" tumors.*
- Individual cells that have already been morphologically classified cannot be measured. Consequently, flow cytometry cannot be used for the DNA cytometric evaluation of urothelial dysplasias.
- *Artifacts* such as nuclear aggregates are indistinguishable from tumor cells.
- *Remeasurement* of the same sample for quality control is *not* possible due to the fading effect.
- Flow cytophotometers usually must be serviced and adjusted by *specially trained personnel.*

10.6.2 Diagnostic Results

10.6.2.1 Identification of Neoplastic Urothelial Cells

Klein et al. (1982) showed that DNA flow cytometry can detect aneuploidy 12–18 months before the appearance of a cystoscopically detectable tumor. These findings demonstrated the possibility of early cancer diagnosis by flow cytometry. Devonec et al. (1982), in tests on 110 bladder irrigation specimens from patients with conservatively treated low-stage bladder tumors, found that DNA flow cytometry was more sensitive than conventional subjective cytodiagnosis in detecting cytologic abnormalities. All 34 samples with positive cytology were diagnosed as atypical by flow cytometry, and there were an additional 39 cases in which only flow cytology was positive.

Generally, success in the detection of aneuploid DNA distributions correlates closely with the tumor stage and grade of malignancy: T0=0%, T1=27%, T2=71.4%, T3/4=75%; G0=0%, G1=30%, G3=77.0% (Chin et al. 1985).

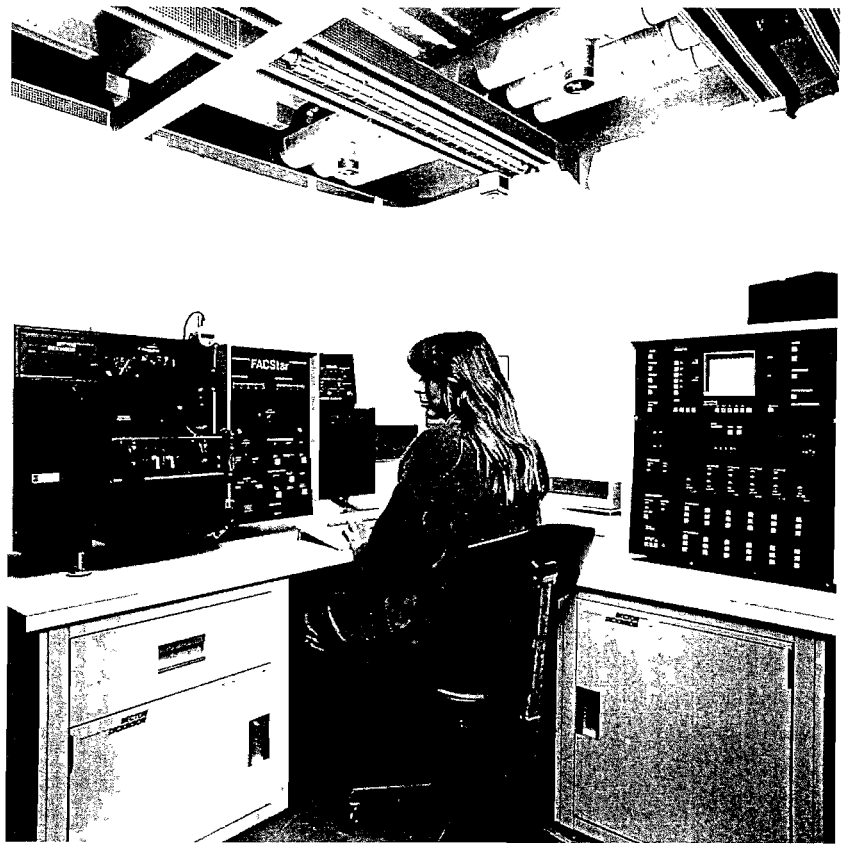

Fig. 10.12. **FACStar Plus flow cytometer with cell sorter** (Becton-Dickinson, USA)

The average sensitivity of the DNA flow cytometric detection of aneuploidy in bladder irrigation specimens from bladder cancer patients as reported in the literature (Table 10.2) is 79%. Badalament et al. (1988) report an average sensitivity of 87% in a review of all 462 bladder carcinomas measured at the Memorial Sloan Kettering Cancer Center in New York. The average sensitivity of urinary cytology according to these studies is only 68.7%. By combining both methods, the sensitivity can be increased to 93%. The average specificity of DNA flow cytometry in bladder washings is 92% as reported in the literature, versus 97% for urinary cytology (see Table 10.2).

Thus, the sensitivity of urinary DNA flow cytometry exceeds that of conventional cytometry by more than 10%. Because the diagnostic capabilities of both methods are supplementary, they should be applied in an adjunctive rather than competitive fashion. Because the specificity of flow cytometry is compromised by a relatively high (8%) rate of false-positive findings, especially in patients with inflammations and stone disease, a positive DNA flow cytometric diagnosis should always be substantiated by another diagnostic technique. Tanke et al. (1983) suggested that DNA image cytometry would provide an accurate check when performed on abnormal nuclei that have been selected out by flow cytometry with a cell sorter, but this would be too costly for practical use.

10.6.2.2 Discriminating Benign and Malignant Dysplasias

Flow cytometry is poorly suited for this task because it does not permit the selective DNA measurement of cells previously diagnosed as dysplastic by morphologic evaluation. However, flow cytometry can often detect aneuploid cells in cystoscopically normal-appearing bladder mucosa that is located near an overt tumor (Hostmark et al. 1984; 54%). This provides an index for systemic tumor involvement of the urothelium.

10.6.2.3 Grading of Malignancy

Although DNA flow cytometry can scarcely do more than discriminate "diploid" from aneuploid tumors, this differentiation in itself has significant prognostic implications. Tribukait and Esposti (1978) found a correlation between histologic tumor grade, the occurrence of invasion, and the fre-

quency of aneuploid cell populations. Later these authors (Tribukait et al. 1979) showed that the percentage of cells in the S phase is also prognostically significant. In 229 operatively treated patients with stage Ta/T1 bladder cancers, this same group (Gustafson et al. 1982a) showed that progression occurred exclusively in tumors with an "aneuploid" DNA distribution pattern, whereas none of the "diploid" tumors exhibited progressive behavior. Similar results were reported by deVere White et al. (1988a,b).

In patients with carcinoma in situ, more rapid progression was observed when more than one aneuploid stemline was detected (Gustafson et al. 1982b). There are no published reports of a correlation between DNA flow cytometric results and length of survival in patients with bladder carcinoma. This offers further evidence that DNA flow cytometry permits only a relatively gross prognostic evaluation of bladder carcinomas.

Bretton et al. (1989) used flow cytometry for monitoring therapeutic response in bladder cancer patients treated with BCG. While a negative examination was interpreted as evidence of therapeutic response, a positive result was considered a predictor of tumor progression.

Thus, rapid DNA flow cytometry appears to have value for bladder carcinoma screening in high-risk groups, for predicting invasive growth in Ta/T1 carcinomas, and for identifying tumor recurrence and confirming response to conservative treatment. However, all the prognostic information derived from DNA flow cytometry can also be furnished by DNA single-cell cytometry. The latter procedure, moreover, usually permits a finer prognostic evaluation.

Through the addition of further quantitative parameters to flow cytometry, it is hoped that the sensitivity and specificity of this procedure can be increased (Ley et al. 1989; Wright et al. 1989).

10.7 Automated Systems

It has been an expressed goal of cytoautomation to develop machines for the automatic prescreening of smears from the uterine cervix. With minor software modifications, these devices can also be used for the analysis of urinary sediment. Automated systems should assume the function of a cytologic assistant in the prescreening of cell smears, and do so at a consistently high performance level that is not degraded by fatigue. Additional requirements are a false-positive rate <10%, a false-negative rate <5%, the ability for a cytologist to check the machine diagnosis in the same specimen, a processing time of about 5 min or less per specimen, a processing rate of approximately 20 000 specimens per year, and a procurement cost below 500 000 DM (Sprenger 1985).

Koss' work with the TICAS system (Taxonomic Intra-Cellular Analytic System) achieved no better than a sensitivity of approximately 81% and specificity of approximately 78% using manual cell selection (Koss et al. 1975, 1978a,b, 1980). Similarly, use of the SAMBA system has not yet produced results that satisfy the requirements of an automated cytometric system (Brugal et al. 1986).

One development that could find use both for cytoautomation and for cytometrically assisted diagnosis is the CAESAR system (Gahm and Aikens 1990). Preliminary results in 63 urine samples indicate a sensitivity of 75% and specificity of 80%. This system employs a Kontron IBAS 2 image analysis system. The CAESAR system is not yet marketed commercially as a software-hardware unit.

The most advanced automated system at present is the LEYTAS II system (or MIAMED-ACA) manufactured by the Wild-Leitz Co. of Wetzlar, Germany (Ploem et al. 1979). Sensitivity/specificity levels of 100%/83.5%,

Table 10. 2. **Accuracy rates of the conventional and DNA flowcytometric diagnosis of urothelial carcinoma in spontaneously voided urine according to reports in the literature**

Authors	Cytologic sensitivity (%)	Flow cytometry		Cytology and flow cytometry sensitivity (%)
		Sensitivity (%)	Specificity (%)	
Klein et al. (1982)		93 (n=208)	98 (n=100)	
Murphy et al. (1986)	75 (n=105)	78 (n=105)	78.6 (n=28)	95 (n=109)
Badalament et al. (1986)	59.1 (n=60)	80.3 (n=66)		
Badalament et al. (1987)	61(n=70)	83 (n=70)		
Jitsukava et al. (1987)	43 (n=56)	73 (n=56)		80 (n=56)

98.3%/88.4%, and 92.4%/96.7% have been determined for the automated screening of smears from the uterine cervix (Ploem 1989). Tanke et al. (1982) used the system for the automated screening of urine samples and achieved a sensitivity of 92.3% with a specificity of 84.1%. With the recent decision of the Wild-Leitz Co. to discontinue the development of image analysis systems for medicine, valuable developmental work invested in this outstanding system has been lost. There is little reason to expect that high-performance automated cytometric systems will be offered on the European market in the foreseeable future. The routine clinical use of automated systems is further constrained by the costly and involved preparatory procedures needed to produce cellular specimens compatible with automated analysis. As a result, routine smears cannot yet be processed on automated systems with satisfactory effectiveness. Until practical, cost-effective, automatic cytopreparatory techniques become available, the routine use of automated cytometric systems for mass screening will remain an elusive goal.

Automated systems do have a limited role in analyzing the DNA distribution in tumor specimens for the purpose of determining prognosis (Stöckle et al. 1987). This task can be performed equally well by economical interactive systems, though these may require a somewhat greater investment of time. This drawback is offset, however, by the simpler technique of specimen preparation for interactive measurements. It is imperative that the pathologist be able to check at any time on the actual cell population that is being measured. Today these systems are incapable of identifying specific cell populations like normal urothelial cells or connective-tissue cells as such with any degree of accuracy. Automated systems are not yet able to process diagnostically questionable routine specimens with dysplasias or borderline lesions and classify them as malignant or benign by means of DNA cytometry.

10.8 Indications for Diagnostic DNA Cytometry

DNA measurements in urothelial carcinoma cells can provide more objective, more reproducible, and usually more prognostically relevant information than a conventional histologic or cytologic evaluation with malignancy grading. Thus, diagnostic DNA measurements are indicated whenever an accurate prognostic assessment can influence further diagnostic and therapeutic procedures. For example, the physician can use the DNA malignancy grade as a guide for determining further course of action following the transurethral resection of a superficial bladder carcinoma. If the DNA malignancy grade is low, a wait-and-see approach is justified, whereas a high DNA malignancy grade may warrant adjunctive chemotherapy. Today, DNA measurements for prognostic purposes are obtained most easily, economically, and precisely by means of interactive single-cell cytometry. This can be performed on any tumor-cell-positive urine sample or washing and even on biopsy material. Since modern TV image analysis systems can measure a cell nucleus in less than 1 s, larger numbers of cells, which previously were reserved for flow cytometers, can be measured within minutes. But TV image analysis is superior to flow cytometry in that the examiner knows which cell populations have been measured. The measurement of hypocellular samples (<10 000 cells) is also possible by single-cell cytometry.

In many cases of urothelial dysplasia, DNA cytometry also can establish a diagnosis of "prospective malignancy" by the detection of aneuploid cells. This provides a means for the further and possibly definitive investigation of cytodiagnostic problem cases. Only interactive DNA single-cell cytometry is appropriate for this task, as it enables the cytologist to individually identify and classify the questionable cells.

DNA flow cytometry offers a way to increase the generally unsatisfactory sensitivity of conventional urinary cytology. Centers that possess such a system and the personnel to operate and maintain it can use DNA flow cytometry as a rewarding adjunct to urinary cytology, especially for the screening of high-risk subjects. Due to the high rate of false-positive diagnoses in urine and bladder washings, however, the detection of aneuploidy by flow cytometry should always be corroborated by other investigations.

Automated systems for the automatic screening of urinary sediment are still in the developmental stage. They are inappropriate for the determination of prognostic DNA parameters in urothelial cells, which can be obtained more economically with interactive systems.

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