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REVIEW

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The Feulgen reaction 75 years on

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Abstract The Feulgen reaction proposed by Feulgen and Rossenbeck 75 years ago is one of the cytohistochemical reactions most widely used in biology and medicine. It allows DNA in situ to be specifically stained based on the reaction of Schiff or Schiff-like reagents with aldehyde groups engendered in the deoxyribose molecules by HCl hydrolysis. The staining intensity is proportional to the DNA concentration. Current applications of the Feulgen reaction are mainly concerned with DNA quantification in cell nuclei by image cytometry for ploidy evaluation in tumor pathology. From the morphological point of view, specific demonstration of DNA in cell structures at the light microscopic level is very little used nowadays. On the other hand, application of the Feulgen principles to electron microscopy have recently allowed specific DNA-staining procedures to be developed for the study of the structural organization of DNA in situ.

Introduction

The Feulgen nuclear reaction for the specific staining of DNA in cytohistochemical samples in situ was introduced by Feulgen and Rossenbeck in 1924. These authors devised a chemical treatment, HCl hydrolysis, to produce free aldehyde groups in the DNA backbone structure that could be detected by a colored reaction for aldehydes (Fig. 1) developed 58 years before (Schiff 1866). Since its introduction, the Feulgen reaction has remained one of the most widely used cytohistochemical reactions in biology and medicine.

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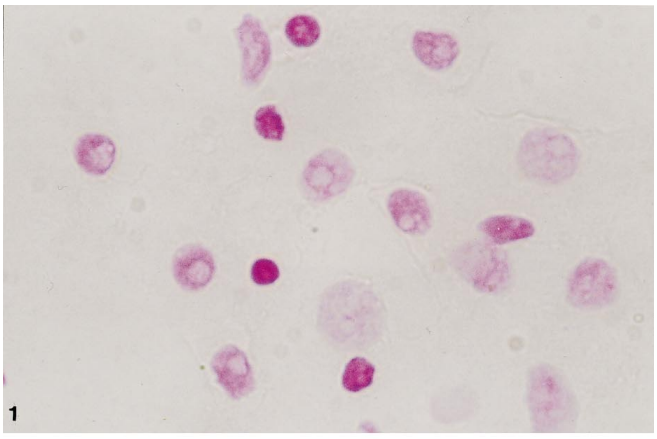
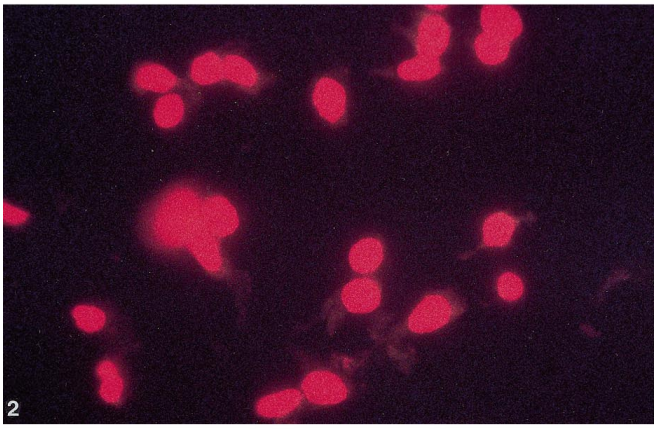
The history of the Feulgen reaction spans almost all of the twentieth century. During this time the technique has served fundamental objectives in the biological and biomedical sciences concerning location and quantitative distribution of DNA in a variety of normal and abnormal cells. In particular, the Feulgen reaction helped to establish that: (1) a 1:1 relationship exists between the DNA content of a nucleus and its chromosome number (Vendrely and Vendrely 1948), (2) the DNA content is the same, or falls into classes that are multiple integers of two, in all the cell nuclei of a given organism (Swift 1950), and (3) before mitosis cells double their DNA content (Patau and Swift 1953). The Feulgen reaction also first permitted measurement of the amount of DNA present in single chromosomes (Gaillard et al. 1968; Nitsch et al. 1970) and comparison of the nuclear DNA contents of different botanical and animal species (Atkin et al. 1965; Tiersch et al. 1989).

On the biomedical side, it was soon realized that the Feulgen reaction could be of great utility to reliably measure the DNA ploidy of cells in tumors (Atkin 1954; Atkin and Richards 1956; Miner and Kopac 1956). The diagnostic and prognostic usefulness of DNA ploidy measurement was definitively assessed for several neoplastic lesions (for review see Atkin 1979; Caspersson 1979; Sandritter 1979; Hall and Fu 1985; Mellin 1990). A large body of research on abnormalities in DNA ploidy and cell cycle analysis was also addressed to a variety of non-neoplastic pathologies (Lapham 1962; Kompmann et al. 1966; Grove et al. 1976; Barrett et al. 1983; Adler and Friedburg 1986). Finally, application of the Feulgen reaction to electron microscopy provided a unique tool to investigate the structural organization of the DNA-containing structures in situ (Derenzini 1995).

As we observed above, on the chemical side the Feulgen reaction is based on quite simple principles and is sufficiently specific for scientific use. However, in the last 50 years a considerable number of studies have been dedicated to assess the accuracy of the reaction at the cellular level and its reliability or pitfalls in its various applications. Today, several technical aspects of the reac-





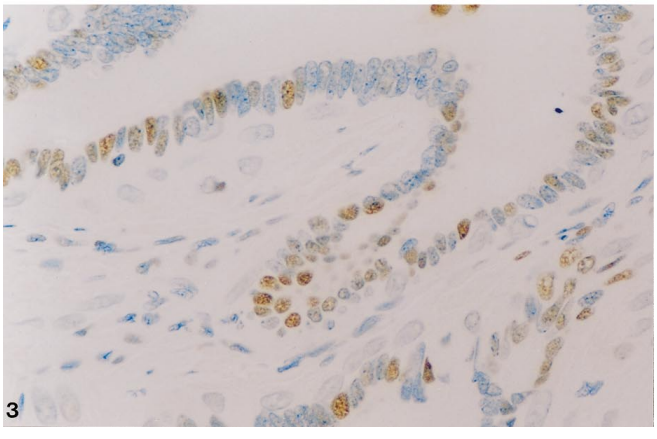


Fig. 1 Cells from a human hepatocellular carcinoma stained with the Feulgen reaction: hydrolysis at 20°C for 45 min, 0.1% pararosaniline-Schiff reagent, pH 1.5, at 20°C for 60 min. Visible light. ×2200

Fig. 2 Cells from a human ovarian carcinoma stained with the Feulgen reaction: hydrolysis at 20°C for 45 min, 0.1% pararosaniline-Schiff reagent, pH 1.5, at 20°C for 60 min. Excitation filter 470–490 nm, barrier filter 630 nm. ×1600

Fig. 3 Section of a human ovarian carcinoma immunostained for proliferating cell nuclear antigen and counterstained with a Feulgen reaction using a Schiff-type reagent containing 0.5% toluidine blue. ×660

tion are still widely discussed. Here, we report the established procedure and mechanism of the Feulgen reaction together with the current applications of the Feulgen reaction in biology and medicine.

The Feulgen reaction

Acid hydrolysis

The mechanism of the primary step in the Feulgen reaction, the acid hydrolysis, is straightforward. In a mild acid environment purine bases are detached from the deoxyribose sugar exposing free aldehyde groups and leaving intact the DNA backbone. Therefore, the DNA molecule becomes “apurinic”. Optimal hydrolytic conditions for the Feulgen procedures have been extensively studied since 1932 (Bauer 1932; Hillary 1939) when it was found that, as hydrolysis proceeds, the DNA filament starts breaking and that this process is influenced by fixative and DNA accessibility. Thus, the production of aldehyde groups may be counterbalanced by a loss of DNA fragments from the nucleus that disturbs the stoichiometry of the reaction (Decosse and Aiello 1966; Rasch and Rasch 1973; Kjellstrand 1977a). In the original procedure, Feulgen and Rossenbeck (1924) suggest that cells should be treated for 4 min with 1 N HCl at 60°C. However, it was later established that for most applications the most favorable hydrolytic conditions are produced with 5 N HCl at 20–25°C for 45–60 min on either unfixed or fixed cells (Kjellstrand 1977b; Böcking et al. 1995). This process delays and minimizes the production of DNA fragments small enough to escape from the nucleus. Nevertheless, both the amount of aldehyde groups produced and DNA solubilization are variably influenced by fixation and chromatin compactness (Duijndam and Van Duijn 1975).

Reaction with the Schiff stain

Following the hydrolytic step, cells are exposed to the widely used Schiff reagent (Schiff 1866; Hörmann et al. 1958; Kasten 1960). Cellular sites with free aldehyde groups in the apurinic DNA molecule bind the bleached pararosaniline dissolved in the Schiff reagent, acquiring a magenta color (Fig. 1). Pararosaniline is the unmethylated form of a series of triaminotriphenylmethane dyes grouped under the names “Magenta” or “basic fuchsin”. It is classified as C.I. basic red 9 (C.I. 42500) and, although not regulated by government agencies, is categorized as “reasonably anticipated to be a human carcinogen” (National Toxicology Program 1998) and must be handled with adequate protection.

The Schiff reagent is essentially prepared by bubbling SO_2 through a 0.5% solution of pararosaniline chloride ($C_{19}H_{18}N_3Cl$; MW 323.82) until saturation. Potassium (or sodium) metabisulfite ($Na_2S_2O_5$), added in the amount of 5–10 mg/ml to a 0.5% solution of pararosaniline acidified with HCl (10 ml 1 N HCl/100 ml final so-

lution), is commonly used today as a source of SO_2. All solutions are finally filtered through 2–10 mg/ml activated charcoal for 1 or 2 min to decolorize the reagent by removing impurities. The final solution should have a pH around 1.5 (Mikel et al. 1985) and be limpid and colorless. Pararosaniline bleaches because SO_2 binds to the chromophoric central double bond destroying the quinonoid structure of the molecule and forming a sulfonic acid compound, which is known as leucofuchsin or leucosulfonic acid.

Chemistry of the reaction

The conjugated double bonds, which confer the red–purple color to pararosaniline, are rapidly restored by the reaction of aldehydes with excess SO_2 in the reagent to give an alkylsulfonic acid that reacts with the amino groups of the leucofuchsin. The central SO_2 is dislodged and the central double bound restored along with the color. An alternative hypothesis suggests that the SO_2 also reacts with the amino groups of the benzene ring to produce an *N*-leucosulfinic acid. The latter reagent would form *N*-sulfinic acid bridges with added aldehydes causing a rearrangement of leucosulfinic acid into a colored quinonoid compound. The structural chemistry of leucofuchsin and of the sulfonic acid–aldehyde link have been debated in several specific studies (Kasten 1960 Puchtler et al. 1975; Gil and Jotz 1976).

Schiff-like reagents

Owing to the high stability of the aldehyde–SO_2–NH complex, a number of studies were undertaken to explore new reagents that could replace pararosaniline in the Schiff reagent (Van Duijn 1956; Kasten 1958; Schwarz and Wittekind 1982). These studies were essentially dictated by the need to find a final reaction product that is more fluorescent than pararosaniline-Schiff or that displays a different color hue for histology, and to study the kinetics of the reaction with a Schiff-type reagent with only one reactive *N* site (Kasten 1960). All the dyes considered as reliable Schiff-like reagents contained at least one free amino group reactive with SO_2 and tested positive for the Feulgen reaction. The fluorescent acriflavine (Böhm and Sprenger 1968; Takamatsu et al. 1980; Mikel and Becker 1991) and the blue-shaded thiazines, thionin (Böcking et al. 1995), and toluidine blue (Chieco et al. 1993), are nowadays the most common substitute for pararosaniline in Schiff-type reagents for quantitative work or simple nuclear counterstaining. Notably, although these reagents have been found specific for apurinic DNA, their reaction mechanism and the structure of reaction products are not yet defined and may significantly differ from that of the original Schiff reagent (Gil and Jotz 1976). Not all Schiff-type reagents yield a true leuco (colorless) form. Thus, they cannot be purified with activated charcoal and poststaining washing steps with acid alcohol or metabisulfite are mandatory to remove non-specific staining from cells and tissues.

Specificity of the reaction

The Feulgen reaction has been severely scrutinized since 1924 by several authors for its specificity and by the end of the 1940s it had already become one the most investigated chemical reactions for intracellular DNA quantification. A broad consensus was created among cytochemists that, under properly controlled conditions, the Feulgen reaction is specific for DNA and no extraneous aldehyde groups are formed in the nuclei (for review see Lessler 1953; Kasten 1960; Pearse 1985). The evidence for this statement came from the following observations: (1) chromosomes do not act as simples adsorbents for the Schiff reagent, (2) the Feulgen reaction can be effectively hindered by blocking aldehyde groups of hydrolyzed DNA by several different aldehyde-coupling reagents, (3) after tissue digestion by deoxyribonuclease the Feulgen reaction does not stain nuclei or chromosomes, (4) 5-min HCl hydrolysis of isolated nuclei not previously fixed does not give rise to DNA solubilization, and (5) prolonged trypsin digestion of chromosomes does not modify the positivity of the Feulgen reaction. Some aspecific dye binding may appear in the cytoplasm and disturb fluorescence measurements. These aspecific reactions are termed “plasmal” (for cytoplasmic aldehydes) when they occur in unfixed cells due to the release of fatty aldehydes from acetal phosphatides (plasmalogens) by the action of $HgCl_2$ or “pseudo-plasmal” when they occur in fixed cells because of the oxidation of unsaturated fatty acids (Takamatsu et al. 1980). Other aspecificities may be found in the cytoplasm of unfixed cells from animals treated with pro-oxidant chemicals, where Schiff-positive aldehyde groups are formed in membranes affected by lipid peroxidation (Pompella et al. 1987). For the Feulgen reaction to be specific, tissue fixation must be appropriate. It is obviously mandatory to avoid aldehyde fixatives such as acrolein or glutaraldehyde which, after fixation, maintain an aldehyde group free to react with the Schiff reagent.

The Feulgen reaction with Schiff and Schiff-type reagents can be conducted in fresh or fixed cells, or in fixed and paraffin-embedded tissues. Various fixation schemes have been found suitable, the most common being 10% buffered formalin for tissues and smears (Delgado et al. 1984; Böcking et al. 1995), and a mixture of methanol (or ethanol), formalin, and acetic acid for air-dried smears (Decosse and Aiello 1966; Böhm and Sprenger 1968; Kiss et al. 1992). Bouin fixatives are not recommended (Mikel et al. 1985).

Current applications of the Feulgen reaction

Nowadays, the Feulgen reaction is used very little for the histochemical demonstration of DNA in cell structures at

the light microscopic level. It is solely applied to ascertain whether a basophilic structure contains DNA. The major exploitation of the Feulgen reaction certainly concerns DNA quantification in cell nuclei by image cytometry for ploidy evaluation. From the morphological point of view, by applying the Feulgen reaction principles to electron microscopy, during the past 20 years specific DNA-staining procedures have been developed that represent unique tools for the study of the structural organization of DNA in situ. These applications of the Feulgen reaction will now be discussed.

Feulgen reaction for the quantitative evaluation of DNA

The possibility of quantifying DNA by the Feulgen reaction is due to the fact that in the Feulgen reaction the color development is proportional to the DNA concentration (Lessler 1953). Early microcolorimetric investigations with gelatine–DNA preparations and the original pararosaniline-based Schiff reagent showed a linear increase in color starting at 0.2 mg DNA/ml. At concentrations higher than 1 mg/ml linearity is lost. However, at DNA concentrations higher than 2 mg/ml, a further slower linear increase occurs (Lessler 1953). It has been shown that the commonly used Schiff and Schiff-type reagents react with all the aldehyde groups formed in the DNA molecule during hydrolysis and that the final color (or fluorescence) intensity is proportional to the number of aldehyde groups released in the DNA molecules (Kasten 1960; Dujindam and Van Duijn 1975).

Considering that in the human diploid nucleus the DNA concentration is quite high, in the order of 10 mg/ml, and that the rate of formation of aldehyde groups in the hydrolysis step is different in nuclei with different degrees of chromatin compactness (Dujindam and Van Duijn 1975), precise measurements of nuclear DNA mass require carefully controlled and standardized reaction procedures ensuring a number of reactive aldehyde groups that are proportional from hypodiploidy to octaploidy and even higher DNA values. Accordingly, it is customary to test the linearity of the reaction by a preliminary measurement of the DNA content in diploid and tetraploid nuclei of various sizes, for example, in lymphocytes, fibroblasts, and hepatocytes (Kiss et al. 1992). Often, a correction factor is needed to match DNA ploidy measurements in nuclei of different sizes and chromatin compactness (Kiss et al. 1992; Böcking et al. 1995).

DNA cytophotometry

The DNA content of nuclei stained with the Feulgen reaction was first quantified by cytophotometry. In situ quantification of DNA was originally performed by ultraviolet (UV) cytophotometry on unstained cell nuclei using microscopes equipped with quartz optics, photographic plates, and a densitometer to measure gray levels on cell micrographs taken under UV light at different wave lengths (Caspersson 1936; Caspersson and Schultz 1939). These first fundamental works, based on the strong UV absorption of nucleic acids, served to indicate that DNA was highly concentrated in the cell nucleus and that high concentrations of RNA were associated with protein synthesis in the cytoplasm. These measurements, however, were cumbersome and expensive and provided data that were difficult to interpret (Swift 1966), because the DNA and RNA absorption curves were overlapping, and thus this method was not applied in many laboratories.

The first microscope photometers designed to measure Feulgen-stained nuclei appeared in 1947 (Pollister and Ris 1947). DNA quantification was much simplified and the Feulgen technique spread very rapidly in all fields of biology both for nuclear and chromosome studies. These instruments mainly worked by positioning the nucleus at the center of a small microscope field that was imaged on a small spot at the photometric window. Absorption measurements were taken under monochromatic light at a wavelength somewhere between 550 and 570 nm, where the pararosaniline-Feulgen-stained DNA has its absorbance peak (Kasten 1960). With these relatively simple instruments it was possible to analyze several related photometric problems (Deeley 1955; Pollister and Ornstein 1959; Swift 1966; Goldstein 1970, 1971, 1975) affecting measurements, so that appropriate procedures, such as the measurement of the stained nuclei with two different wavelengths (Ornstein 1952; Chieco and Boor 1984) or photographic densitometry (Adams 1968), could be designed to reduce photometric errors.

The major obstacle in quantitative DNA cytochemistry was the distributional error (Ornstein 1952; Swift 1966; Goldstein 1971). This error, caused by the non-homogeneous distribution of a particulate chromophore in the nuclei, distorts the measurement so that the absorption measured with a single photometric spot as large as the nucleus, does not correlate with the DNA content. The strategy most widely adopted was devised by Deeley (1955) who proposed raster scanning of each nucleus with a tiny measuring spot (0.25–0.5 μm). The nucleus was then traversed by tenths of individual readings as small as the limit of the objective resolution so that heterogeneities could not be solved. Automatic stage or flying scanning cytophotometers suitable for Feulgen quantification were then developed along the same lines (for review see Zimmer 1973; Altman 1975; Caspersson and Kudynowski 1980; Rost 1980).

Given the fluorescent properties of nuclei stained with the original pararosaniline-Schiff reagent (Fig. 2) and some of the Schiff-type reagents, DNA ploidy can be evaluated also with a microscope cytofluorometer that measures the fluorescence emitted from the nuclei, a type of measurement that is not affected by distributional error(Böhm and Sprenger 1968; Prenna et al. 1974). It is noteworthy that the DNA ploidy of single isolated cells or nuclei stained with Schiff-type fluorescent reagents can be measured using flow cytofluorometers (Kamentsky and Melamed 1969; Kraemer et al. 1971; Trujillo and

Van Dilla 1972), whose first models were developed in the same period that saw the appearance of scanning microscope photometers (Kamentsky et al. 1969; Van Dilla et al. 1969).

DNA image cytometry

In the early eighties, scanning cytophotometers were still very expensive and the less costly single-spot or fluorescence cytophotometric microscopes were accessible only to dedicated research laboratories. The use of these instruments required highly trained technical specialists, not infrequently at the Ph.D. level. Things changed in the second half of 1980s with the advent of image cytometry which, using the rastering capabilities of video cameras, allowed for simpler and cheaper instrumentation (Wied et al. 1983). The relatively new solid-state cameras and the increasing availability of cheap computer power proved to be a stimulus for the development and refinement of the inexpensive instrumentation that is today extensively used to determine DNA ploidy in clinical and research settings (Mellin 1990; Bacus and Bacus 1994; Bocking 1995; Cohen 1996). Recently, interlaboratory standardization and quality control of the Feulgen reaction in either cell sampling, staining, or measurement became a major topic of discussion in meetings and journals devoted to cytometry (Böcking et al. 1995; Marchevsky et al. 1996; Thunnissen et al. 1997).

Today the measurement of DNA ploidy in cells layered on a microscope slide is mostly performed using image cytometers. The video camera is provided with a charge coupled device (CCD) sensor (Blouke et al. 1983; Donovan and Goldstein 1985; Aikens et al. 1989). The CCD is subdivided into thousands of tiny square or rectangular potential wells (6–15 µm side) each behaving as an independent true photometric unit which transforms single photons in photo electrons. Thus, with appropriate enlargement, each nucleus is divided into hundreds of independent single photometric measurements which are summarized to give the final integrated optical density. The basic functioning is comparable to that of the old scanning stage or flying-spot cytophotometers (Altman 1975) with the difference that the scanning is now electronic and no stage or light beam raster movement is necessary. Image cytometers are highly efficient and very easy to use at less than one-third of the cost of previous instruments. Thus, thanks to the advances in image cytometry, the problem of cost and complexity of instrumentation, that for a long time hampered the use of the Feulgen reaction, has today been completely overcome.

Specimen preparation

DNA ploidy with the Feulgen reaction can be measured in nuclear or cellular suspensions spread onto a slide, in cell-cultured plates, in biological fluids, and in histological sections. Isolated intact single cells are by far the most common targets to determine DNA ploidy with the Feulgen reaction. Cells can be dissociated from cell cultures and fresh tissues with methods that loosen cell-to-cell contact and stromal adherence. Suitable cytological preparations can also be easily obtained from blood and other body fluids, such as urine, effusion liquids, or sputum (Keebler 1991), and from isotonic solutions used to wash cavities, biopsies, or surgical specimens (Wohnrath et al. 1995). Touch imprinting of fresh tissue onto a slide (Veneti et al. 1996) and scraping of tissue surfaces (Lee et al. 1993) are additional methods to dissociate single cells from tissue. Once in suspension, cells can be layered onto a slide by cytocentrifugation (Leeman et al. 1971) or first collected in polycarbonate filters with a 3- to 8-µm pore size (Oud et al. 1984; Melchiorri et al. 1994) and then transferred to a slide by gently blotting the filter onto the glass surface. Once layered on a slide, cells are usually fixed before staining procedures are begun.

Single cells can be dissociated from previously fixed or fixed and paraffin-embedded tissues using similar although stronger shriving forces and enzymatic activities than those used for fresh samples. Starting material for cell suspensions can derive from a minced tissue sample (Chieco et al. 1991), a core biopsy punched in a paraffin block (Delgado et al. 1984; van Driel-Kulker et al. 1986), or a thick histological section (Hedley et al. 1983; Stephenson et al. 1986). Several matched studies have shown that the analysis of DNA ploidy of cell populations isolated from fixed tissues gives comparable results to those isolated from matched unfixed tissues (van Driel-Kulker et al. 1985; Bosari et al. 1991). Whereas in flow cytometric studies, cells isolated from formalin-fixed tissue have not been found optimal for DNA analysis since the intercalating fluorescent dyes stain cytoplasmic debris (Albro et al. 1993) and penetrate poorly and unpredictably crosslinked chromatin (Overton and McCoy 1994), the Feulgen reaction actually performs quite well, providing that appropriate internal control cells and correction for difference in chromatin compactness are used (Allison et al. 1981; Schimmelpenning et al. 1990; Kiss et al. 1992).

DNA extracted from formalin-fixed and paraffin-embedded tissue has been found to be extensively and variably degraded so that only small DNA fragments (<200 bp) can be reliably amplified by polymerase chain reaction (Goelz et al. 1985; Iwamoto et al. 1996; Roehrl et al. 1998). The extent of DNA degradation may vary in different organs (Iwamoto et al. 1996). Although DNA depurination by acid hydrolysis occurs either in denatured or degraded DNA, nevertheless these observations indicate that the nuclei from different cells and different preparations containing variously conformed DNA can be unpredictably sensitive to hydrolytic fragmentation. Thus, caution should always be taken in evaluating small ploidy differences measured in Feulgen-stained cells.

Controversies still exist as to whether DNA ploidy can be reliably measured in tissue sections. Numerous studies have been made to find mathematical algorithms

capable of adjusting for truncated nuclei in thin tissue sections with uniform spherical nuclei, such as rat liver (for review see Mairinger and Gschwendtner 1996). Consensus on this topic is still lacking even for such fundamental aspects as the correct tissue thickness, and these algorithms are not in common use when DNA ploidy has to be measured in preneoplastic or neoplastic lesions where nuclear size and morphology vary greatly (for a discussion forum see Chieco and Van Noorden 1996; Cohen and DeRose 1996). Attempts to select intact nuclei from thick tissue sections fail because of nuclear overlapping (Williams et al. 1996) and the impossibility of having the entire nucleus flat on a single focal plane, as required by cytophotometry (Chieco et al. 1994). The major use of the Feulgen reaction in intact tissue sections, particularly using blue thiazines in the Schiff reagent, is to counterstain nuclei (Fig. 3). The absence of cytoplasmic stain is particularly useful in quantitative immunohistochemistry using image cytometry where it is necessary to precisely segment the nuclei on a monitor.

Units of measure

Once measured, the amount of DNA is expressed in "c units" where c stands for "content" or "complement" and "1c" is the nuclear DNA content of a haploid set of chromosomes; therefore a diploid value is denoted "2c". When ploidy is determined in this way, the terms are "DNA ploidy" and "DNA aneuploidy" to differentiate clearly from chromosome counting obtained by cytogenetic techniques (Hiddeman et al. 1984).

Some authors prefer to express DNA ploidy measure as "picograms of DNA" per nucleus (Bacus and Bacus 1994). This requires a previous biochemical measurement (commonly available in the scientific literature) that tells us how many picograms of DNA are present in the nuclei of fresh diploid cells from the same cell population as the one we are measuring or from a reference species (often trout or chicken nucleated erythrocytes and human lymphocytes). Picogram units were used to demonstrate the constancy of the amount of DNA per resting nucleus in the same tissue, organism, and species using different biochemical methods (Thomson et al. 1953). They are absolutely required when we want to compare the DNA content of different species (Atkin et al. 1965; Tiersch et al. 1989), but add unnecessary complexity when working with mixed target and control cells from the same organism, particularly if stem line ploidy or cell cycle disturbances are investigated.

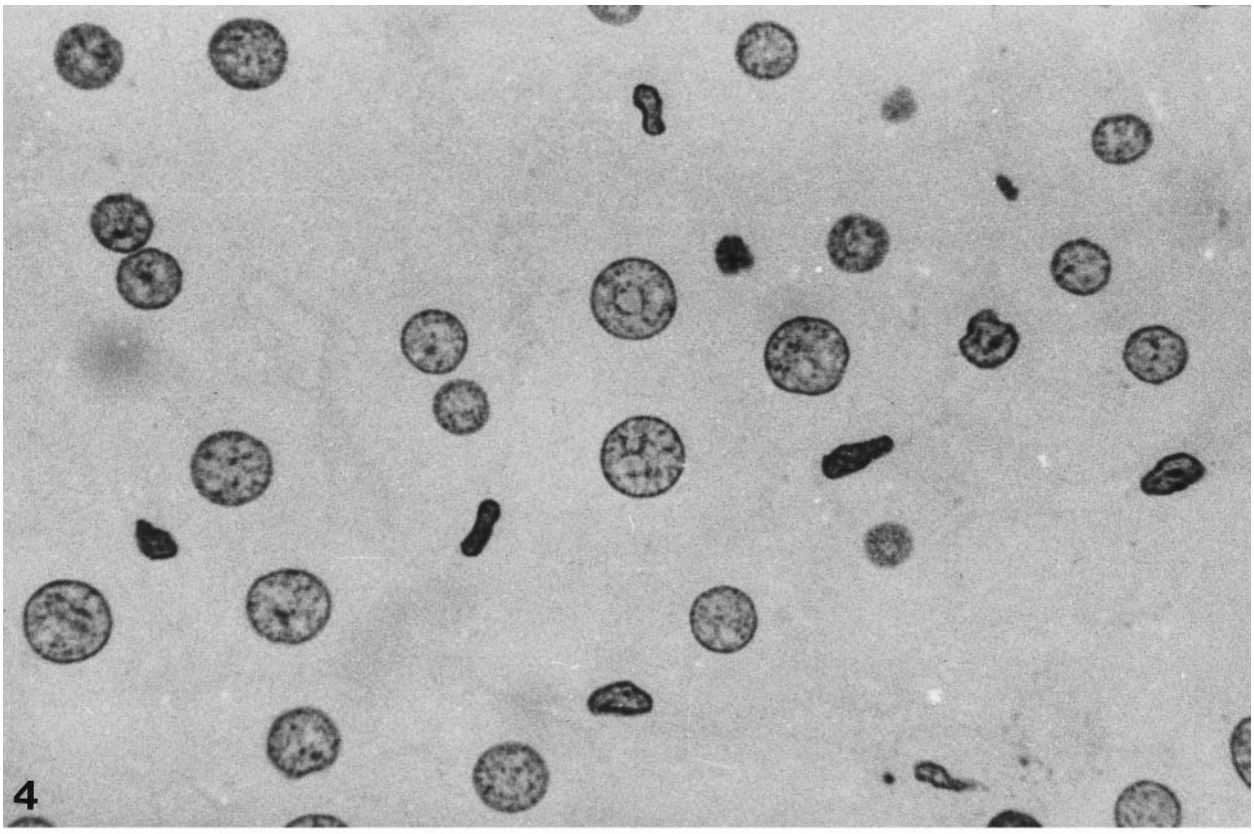
The Feulgen reaction in electron microscopy

Ultrastructural definition of the macromolecular composition of nuclei greatly accelerated after the introduction, during the 1960s, of aldehyde fixatives and water-soluble resins which gave rise to a series of cytochemical methods for the visualization of DNA and RNA (Marinozzi 1966). Among the different approaches proposed for distinguishing DNA from the other macromolecules in situ those based on the principles of the Feulgen reaction are particularly noteworthy, and one of these, the Feulgen-like osmium ammine reaction, can be considered to represent the method of choice for the visualization of the structural organization of the DNA-containing structures in thin sections (Derenzini 1995).

To be visualized at the transmission electron microscope, a substance must be characterized by an intrinsic electron opacity or must be rendered electron opaque by becoming linked to an electron-opaque stain. Adaptation of the Feulgen reaction procedure from light to electron microscopy has been carried out considering four methodological approaches: (1) exploitation of the intrinsic electron opacity of the Schiff reagent, (2) increasing the contrast of the apurinic acidic-Schiff reagent complex by the use of high electron opaque markers reacting with the Schiff reagent, (3) use of silver nitrate solution in reaction with aldehyde groups, and (4) utilization of Schiff-like reagents characterized by high electron opacity. These different technical procedures will be reported separately.

DNA visualization in thin sections using the Schiff reagent

The attempt to specifically visualize DNA in thin sections using the Schiff reagent was applied by Finck (1958) to unfixed, frozen-dried rat tissues before embedding. The chromatin of hepatocytes evidenced an increased electron opacity, whereas the nucleolar body appeared to be much less dense. Similar results were obtained by Yasuzumi and Sugihara (1965) using the same staining procedure in thin sections from Ehrlich tumor cells fixed in formalin and embedded in Epon. Although the electron opacity of the apurinic acid-Schiff reagent complex is not negligible, it is certainly insufficient to study the ultrastructural organization of the DNA-containing structures. However, more recently a very good contrast of the DNA-containing structures was obtained using toluidine blue as the Schiff-like reagent after HCl hydrolysis of thin sections from Lowycril-embedded samples (Derenzini and Farabegoli 1987). After bubbling 0.5% toluidine blue in distilled water with SO_2, the staining solution allows specific visualization of DNA both at the light and electron microscopic levels (Figs. 4, 5) in thick and thin sections, respectively, previously hydrolyzed by HCl. The electron opacity of the staining structures was good enough to clearly visualize very small intranucleolar chromatin clumps. Blockade of aldehyde groups by 10% acetic acid aniline treatment of the sections after HCl inhibited the staining reaction.



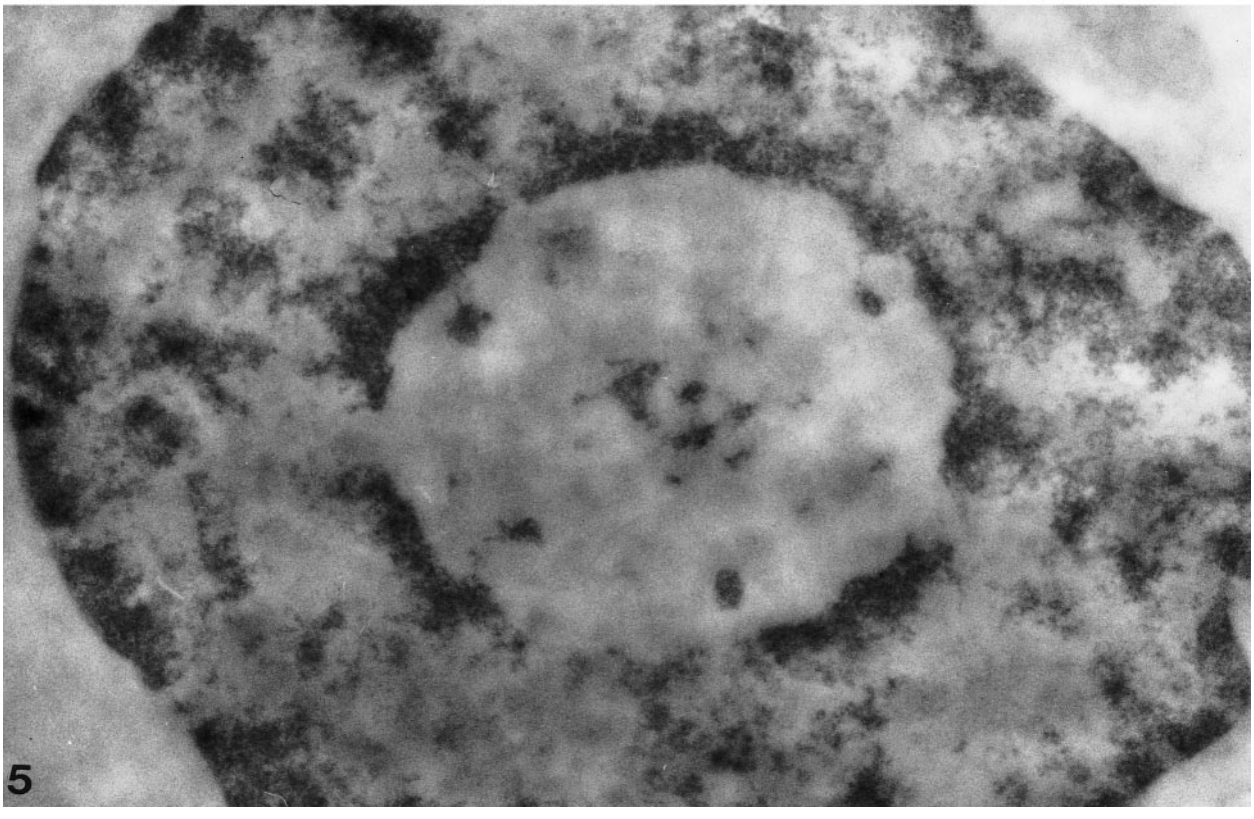


Fig. 4 Semithin section from Lowicryl-embedded rat liver stained with the Feulgen-like reaction with toluidine blue. Only hepatocyte and sinusoidal cell nuclei are stained. ×800

Fig. 5 Thin section from Lowicryl-embedded rat liver stained with the Feulgen-like reaction with toluidine blue. Only the DNA-containing structures are rendered electron opaque in the hepatocyte nucleus. In the electron-translucent nucleolar body, intranucleolar chromatin clumps are clearly visible. ×30,000

DNA visualization in thin sections using electron-opaque markers linking the DNA–Schiff complex

Secondary detection of the Schiff reagent linked to hydrolyzed DNA represents a logical way to increase the electron opacity of the stained structures and, at the same time, to maintain the specificity of the original Feulgen reaction. Phosphotungstic acid was initially proposed for the detection of the Schiff reagent (Thiery 1967). However, as far as the DNA visualization was concerned, the results obtained were discouraging since the staining procedure proved only preferential for DNA, as RNA-containing structures were also revealed (Gautier and Schreyer 1970).

An alternative method for revealing the DNA–Schiff reagent complex using thallium ethylate was developed by Moyne (1973) and this appeared much more fruitful. Thallium ethylate is a reagent for hydroxyl groups (Mentre 1972). The combination of the Schiff reagent with apurinic acid implies the formation of hydroxyls at the level of the complex (Moyne 1973). Therefore, after blockage of the hydroxyls present on other molecules, it is possible to specifically reveal the apurinic acid–Schiff complex by thallium ethylate which is highly electron opaque. The best results were obtained by en bloc acetylation of glutaraldehyde-fixed material before Epon embedding and HCl hydrolysis of thin sections, followed by treatment with the Schiff reagent and thallium ethylate (Moyne 1973, 1974). The staining end reaction product was thin enough to obtain a very good resolution of DNA-containing structures in mammalian cells, plant cells, viruses, and bacteria (Moyne 1980). The Schiff-thallium procedure was actually a Feulgen-like reaction since negative results were obtained in the absence of the Schiff reagent. At the time of its presentation, this technique appeared to be very promising for the study of DNA distribution and organization in electron microscopy. However, a series of drawbacks limited its diffusion. In fact, the staining specificity depends on the previous complete blockage of hydroxyl groups, the blocking of the hydroxyl groups by acetylation must be carried out en bloc, thallium ethylate must be handled in moisture-free conditions, and the staining reaction was not always reproducible. For these reasons, Moyne himself abandoned the Schiff-thallium reaction for a more simple and highly specific DNA staining, the Feulgen-like osmium ammine reaction which had also been proposed at about the same time (Cogliati and Gautier 1973).

DNA visualization in thin sections using silver reagents for aldehyde groups

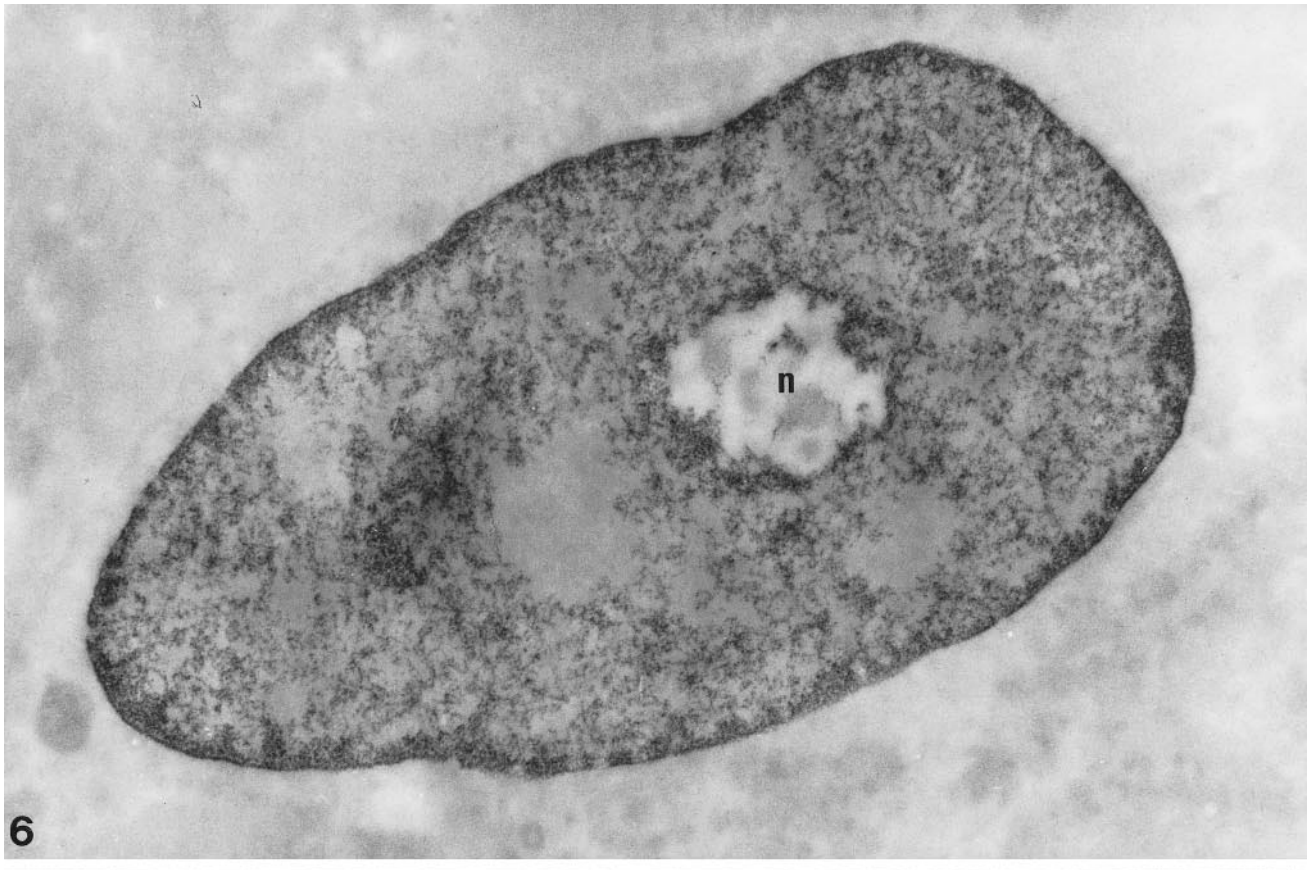
This approach is based on the use of silver salts that are directly visible at the electron microscope. From the historical point of view, the "Feulgen-silver reaction" with an ammoniacal silver nitrate solution to reveal aldehyde groups engendered by HCl hydrolysis initiated the ultrastructural cytochemistry of DNA (Bretschneider 1949). A gross silver precipitate was observed on the bull sperm heads where chromatin is localized. Many authors had then tried to improve this "Feulgen-silver reaction" for the visualization of DNA at the ultrastructural level. Of these, Peters (1966) obtained the best results. The reaction was carried out on thin sections from aldehyde-fixed, Epon-embedded samples using a silver methenamine reagent to reveal aldehyde groups produced by HCl treatment. Silver particles about 2 nm in size were shown to be located on DNA-containing structures, in viruses, in kinetoplasts of trypanosomes, in mitochondria, and in bacteria (Peters 1966; Peters and Giese 1970). The staining reaction was abolished if the pseudoaldehyde groups engendered by DNA hydrolysis with HCl were blocked by aniline-HCl treatment. The "Feulgen-silver reaction", however, was demonstrated to be not truly specific and, as a consequence of the coarse end reaction product, not utilizable for high resolution studies. These drawbacks accounted for the abandonment of this Feulgen technique for DNA visualization at the ultrastructural level.

DNA visualization in thin sections using electron-opaque Schiff-like reagents

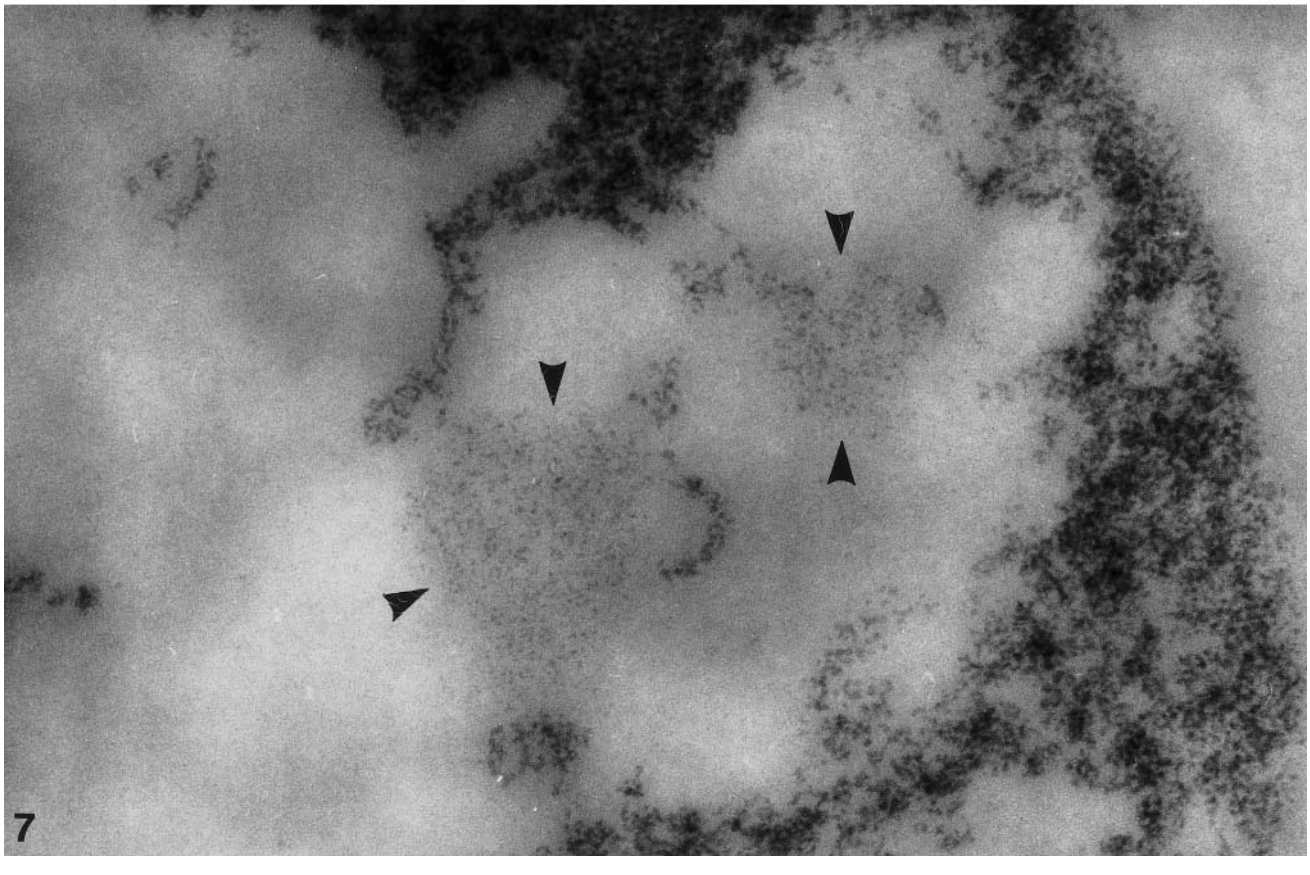
In the late 1960s and early 1970s, Gautier and his research group undertook systematic experiments in order to find electron-opaque Schiff-like reagents that would provide an electron stain exactly parallel to the Feulgen nuclear reaction (Gautier 1976). As defined by Kasten (1960), a Schiff-like reagent must fulfil the following criteria. It must give DNA or glycoconjugate reaction after HCl hydrolysis or periodic acid treatment, respectively, no reaction without HCl hydrolysis or periodic acid treatment, a positive reaction only after SO_2 treatment of the stain, and at least one -NH_2 group must be present in the reagent. During the search for an electron-opaque Schiff-like reagent, Gautier and Schreyer (1970) demonstrated that, after SO_2 treatment, ruthenium red, an inorganic ionic complex between ruthenium atoms and ammoniacal groups, did in fact behave as an electron-opaque Schiff-like reagent giving rise to specific DNA staining in thin sections after HCl hydrolysis. Nevertheless, the low contrast of the stained structures and the "hazardous" reproducibility of the staining reaction, prompted Gautier and coworkers to abandon ruthenium red and to prepare a chemical analogue containing osmium, which shares chemical properties with ruthenium and has an atomic weight of 190, instead of ruthenium which has an atomic weight of 101. The authors succeeded in synthesizing an "inorganic ammine of osmi-

Fig. 6 Feulgen-like osmium ammine reaction. Smooth muscle cell. Only the structures containing DNA are stained in the nucleus; no cytoplasmic organelles are stained. *n* Nucleolus. ×16,000 ▶

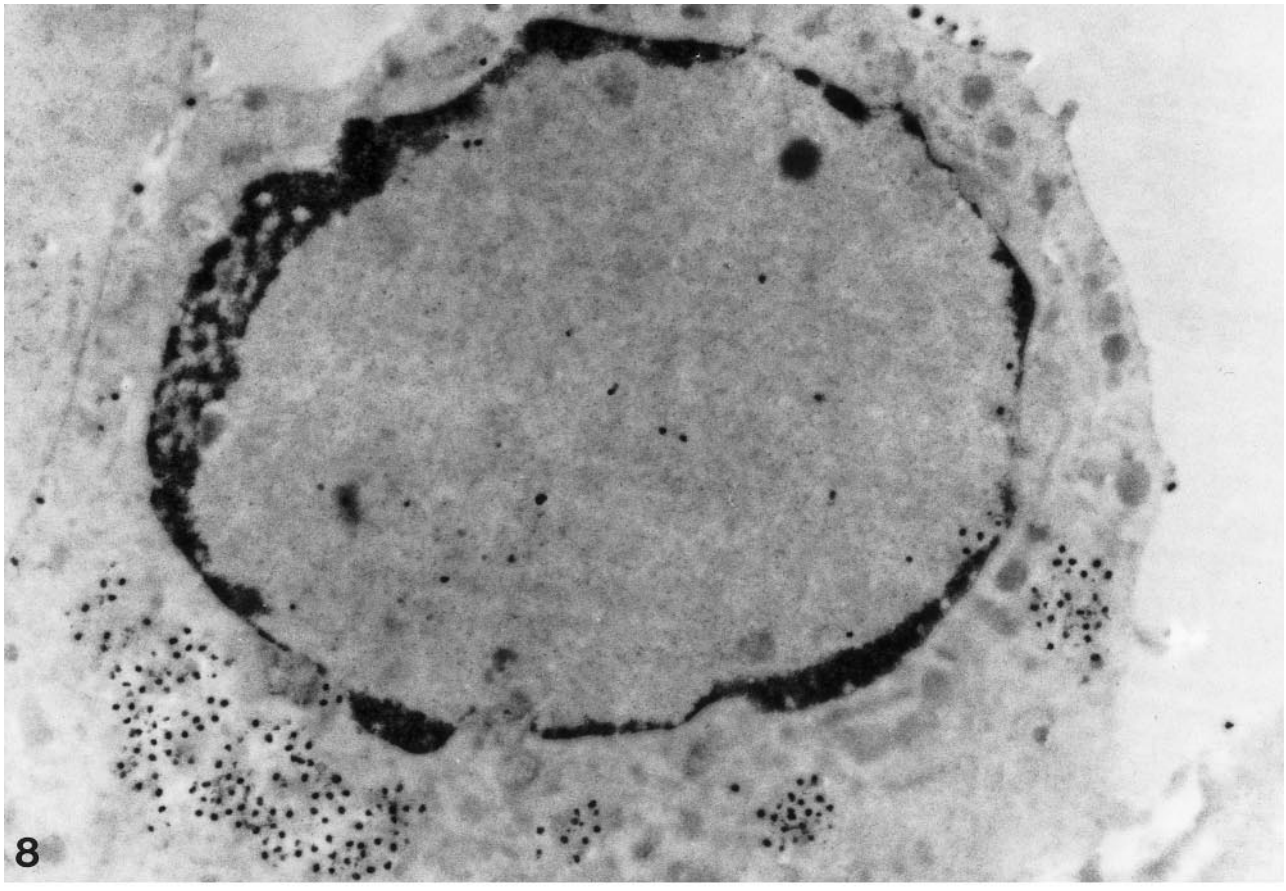
Fig. 7 Feulgen-like osmium ammine reaction. Smooth muscle cell. The intranucleolar chromatin appears to be composed of fibers and loose agglomerates of very thin structures (*arrowheads*). ×150,000



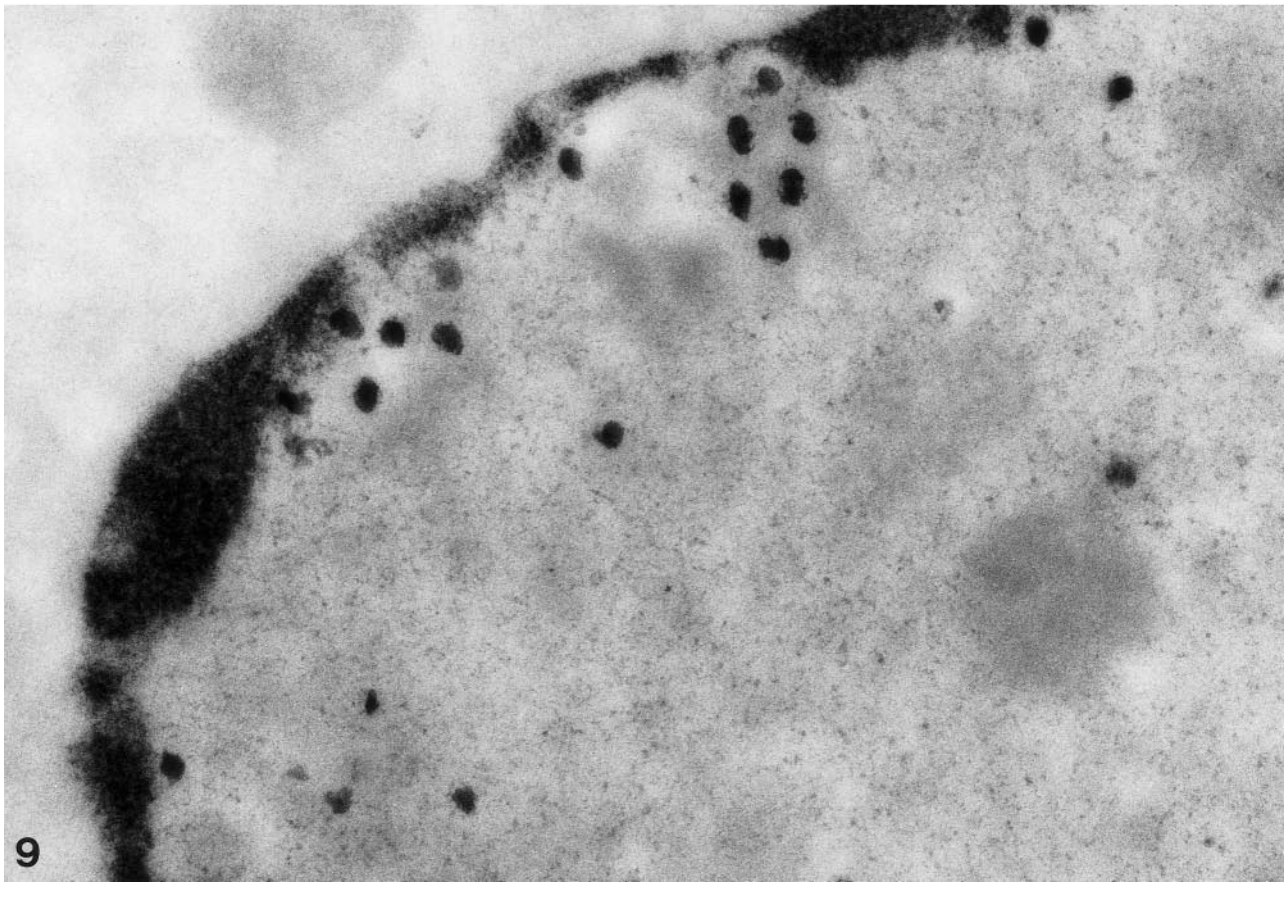
6



7



8



9

um" that when treated with SO_2 fulfilled all the criteria for a Schiff-like reagent (Cogliati and Gautier 1973).

The protocol used by Cogliati and Gautier (1973) to synthesize osmium ammine was not a standardized procedure, presented several difficulties, and the product was not always utilizable. For a long time this was a limiting factor for the widespread utilization of this Schiff-like reagent in electron microscopy. In 1989, Olins et al. succeeded in synthesizing an osmium ammine complex (osmium ammine B) in a highly reproducible manner which exhibits the same cytochemical properties as the original osmium ammine. This new osmium ammine complex has become commercially available and is now routinely employed. The chemical nature of the osmium ammine complex has not been exactly defined; several different osmium ammines and/or polyammine complexes are involved.

Osmium ammine as a Schiff-like reagent in electron microscopy. Osmium ammine/SO_2 is prepared by bubbling SO_2 for 20 min through a 0.2% solution of osmium ammine B in water. Osmium ammine/SO_2 can be used for specifically revealing pseudoaldehyde groups engendered in DNA after HCl hydrolysis or in glycoconjugates after periodic acid treatment. For the specific visualization of DNA or glycoconjugates no other aldehyde groups must be present in the sample apart from those produced by HCl or periodic acid treatment. This is very important for the choice of the fixative. The two more widely employed fixatives in electron microscopy are osmium tetroxide and glutaraldehyde. Osmium tetroxide is obviously excluded as it confers an intrinsic electron opacity to the fixed substances, and also glutaraldeyde cannot be used as it leaves an unsaturated aldehyde group in the fixed material which reacts with the osmium ammine/SO_2 complex. Formaldehyde, which engages in the fixation reaction the only one aldehyde group of the molecule, is therefore recommended (Derenzini et al. 1982). The staining reaction can be carried out in thin sections from frozen, Lowycril, LR white, and Epon-embedded samples. After HCl pretreatment of the sections, osmium ammine/SO_2 specifically renders electron opaque the DNA-containing structures whereas, after periodic acid pretreatment, only the glycoconjugates are stained. If HCl or periodic acid pretreatment is omitted, the osmium ammine/SO_2 complex does not stain any structure in thin section from formaldehyde-fixed, Epon-embedded material. Staining by osmium ammine/SO_2 complex is prevented if, after HCl or periodic acid pretreatment, blockage of aldehyde groups engendered in thin section is carried out by the aniline-HCl technique or by the aniline-glacial acetic acid method (Moyne 1980). Finally, osmium ammine without SO_2 confers only a very faint contrast to untreated or HCl and periodic acid pretreated sections from Epon-embedded samples (Derenzini et al. 1982). All these data clearly indicated that the osmium ammine/SO_2 complex is actually a Schiff-like reagent.

The osmium ammine Feulgen-like reaction allows the organization of the DNA-containing structure to be defined in situ. The end reaction product of the Feulgen-like osmium ammine staining does not pose any limit to the resolution of the stained structures in thin sections. DNA filaments as thin as a DNA double helix molecule (2–3 nm) have been clearly and repeatedly visualized (Derenzini 1995). A lot of studies have been performed to reveal DNA structure in eukaryotic cells, mitochondria, bacteria, and viruses (Biggiogera et al. 1996). As far as eukaryotic cells are concerned, as shown in Fig. 6, only the DNA-containing structures appear to be electron opaque after the Feulgen-like reaction of thin sections from formaldehyde-fixed samples. In the nucleolar body, which is very electron translucent, few stained structures are visible. At high magnification a particulate organization of the chromatin structures has been described, characterized by roundish units with a diameter of about 12 nm, composed of a DNA filament encircling an electron-translucent inner core (Derenzini 1995). This particulate organization of chromatin in situ is consistent with the nucleosome structure as described in in vitro chromatin. Interestingly, this particulate organization is not present in the ribosomal chromatin located in the fibrillar nucleolar components which in fact appears to be composed of extended DNA filaments (Fig. 7).

The Feulgen-like osmium ammine reaction has also been used to investigate the in situ configuration of viral genomes. Important information of the structure of viral DNA was obtained in infections with viruses showing either an intracytoplasmic development, such as pox virus, or an intranuclear development, such as herpes viruses (Puvion-Dutilleul et al. 1996; Figs. 8, 9).

Conclusions

We should like to conclude the present review by reporting what Lessler said about the Feulgen reaction many years ago (1953). "The Feulgen nucleal reaction provided an important tool for studies concerned with the desoxypentose-nucleic acid (DNA) content and distribution in normal and abnormal cells. Although there are several excellent qualitative and quantitative tests for DNA, the nucleal reaction is the most reliable test for DNA in situ". This statement is still valid. Indeed, after its promulgation in 1924 by Feulgen and Rossenbeck, the contribution of the Feulgen reaction to biomedical research has been outstanding. Notwithstanding the vast utiliza-

◀ **Fig. 8** Feulgen-like osmium ammine reaction. BHK cell infected with herpes virus. Intensely stained viral nucleoids are present in the cytoplasm and, to a lesser extent, in the nucleus at whose periphery chromatin, in a very compact pattern, is located. At this magnification no other cell structure appears to be stained. ×14,000

Fig. 9 Feulgen-like osmium ammine reaction. BHK cell infected with herpes virus. In the nucleus non-encapsulated viral DNA appears as a network of very thin filaments. ×90,000

tion of the reaction by many scientists in 75 years, the precise mechanism of reaction has evaded definition.

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References

Adams LR (1968) A photographic cytophotometric method which avoids distributional error. Acta Cytol 12:3–8

Adler CP, Friedburg H (1986) Myocardial DNA content, ploidy level and cell number in geriatric hearts: post-mortem examinations of human myocardium in old age. J Mol Cell Cardiol 18:39–53

Aikens RS, Agard DA, Sedat JW (1989) Solid-state imagers for microscopy. Methods Cell Biol 16:291–313

Albro J, Bauer KD, Hitchcock CL, Wittwer CT (1993) Improved DNA content histograms from formalin-fixed, paraffin-embedded liver tissue by proteinase K digestion. Cytometry 14: 673–678

Allison DC, Ridolpho PF, Rasch EM, Rasch RW, Johnson TS (1981) Increased accuracy of absorption cytophotometric DNA values by control of stain intensity. J Histochem Cytochem 29:1219–1228

Altman FP (1975) Quantitation in histochemistry: a review of some commercially available microdensitometers. Histochem J 7:375–395

Atkin NB (1954) Nuclear size in carcinomas of the cervix – its relation to DNA content and to prognosis. Cancer 17:1319–1399

Atkin NB (1979) Prognostic and significance of modal DNA value and other factors in malignant tumours based on 1465 cases. Br J Cancer 40:210–221

Atkin NB, Richards BM (1956) DNA in human tumors as measured by microspectrophotometry of Feulgen stain: a comparison of tumors arising at different sites. Br J Cancer 10: 769–786

Atkin NB, Mattinson G, Becak W, Ohno S (1965) The comparative DNA content of 19 species of placental mammals, reptiles, and birds. Chromosoma 17:1–10

Bacus JW, Bacus JV (1994) Quality control in image cytometry: DNA ploidy. J Cell Biochem Suppl 19:153–164

Barrett TB, Sampson P, Owens GK, Schwartz SM, Benditt EP (1983) Polyploid nuclei in human artery wall smooth muscle cells. Proc Natl Acad Sci USA 80:882–885

Bauer H (1932) Die Feulgensche Nuklealfärbung in ihrer Anwendung auf Cytologische Untersuchungen. Z Zellforsch 15: 225–247

Biggiogera M, Courtens JL, Derenzini M, Fakan S, Hernandez-Verdun D, Risueno MC, Soyer-Gobillard MO (1996) Osmium ammine: review of current applications to visualize DNA in electron microscopy. Biol Cell 87:121–132

Blouke MM, Janesick JR, Hall JE, Cowens MW, May PJ (1983) 800×800 charged coupled device image sensor. Opt Eng 22: 607–614

Böcking A, Giroud F, Reith A (1995) Consensus report of the ESACP task force on standardization of diagnostic DNA image cytometry. Anal Cell Pathol 8:67–74

Böhm N, Sprenger E (1968) Fluorescence cytophotometry: a valuable method for the quantitative determination of nuclear Feulgen-DNA. Histochemie 16:100–118

Bosari S, Wiley BD, Hamilton WM, Dugan JM, Cook LB, Lee AKC (1991) DNA measurement by image analysis of paraffin-embedded breast carcinoma tissue. A comparative investigation. Am J Clin Pathol 96:698–703

Bretschneider LH (1949) An electron microscopical study of bull sperm. Proc K Ned Akad Wet 52:301–309

Caspersson T (1936) Über den chemischen Aufbau der Strukturen des Zellkernes. Scand Arch Physiol 73:suppl 8

Caspersson T (1979) Quantitative tumor cytochemistry. Cancer Res 39:2341–2355

Caspersson T, Kudynowski J (1980) Cytochemical instrumentation for cytopathological work. Int Rev Exp Pathol 21:1–54

Caspersson T, Schultz J (1939) Pentose nucleotides in the cytoplasm of growing tissues. Nature 143:602

Chieco P, Boor PJ (1984) Quantitative histochemistry in pathology and toxicology. An evaluation of the original two-wave length method of Ornstein. Lab Invest 50:355–362

Chieco P, Van Noorden C (1996) Letter to the editor. Mod Pathol 9:84–85

Chieco P, Melchiorri C, Lisignoli G, Marabini A, Orlandi C (1991) A multifaced DNA ploidy analysis to determine ovarian carcinoma aggressiveness. Cancer 67:1878–1885

Chieco P, Pagnoni M, Romagnoli E, Melchiorri C (1993) A rapid and simple staining method, using toluidine blue, for analyzing mitotic figures in tissue sections. Histochem J 25:569–577

Chieco P, Jonker A, Melchiorri C, Vanni G, Van Noorden CJF (1994) A user's guide for avoiding errors in absorbance image cytometry: a review with original experimental observations. Histochem J 26:1–19

Cogliati R, Gautier A (1973) Mise en evidence de l'ADN et des polysaccharides a l'aide d'un noveau reactif de type Schiff. C R Acad Sci (Paris) Ser D 276:3041–3044

Cohen C (1996) Image cytometric analysis in pathology. Hum Pathol 27:482–493

Cohen C, DeRose PB (1996) Letter to the editor. Mod Pathol 9:86–87

Decosse JJ, Aiello N (1966) Feulgen hydrolysis: effect of acid and temperature. J Histochem Cytochem 14:601–604

Deeley EM (1955) An integrating microdensitomer for biological cells. J Sci Instrum 32:263–267

Delgado R, Mikuz G, Hofstädter F (1984) DNA-Feulgen-cytophotometric analysis of single cells isolated from paraffin-embedded tissue. Pathol Res Pract 179:92–94

Derenzini M (1995) Ultrastructural cytochemistry. In: Morel G (ed) Visualization of nucleic acids. CRC Press, Boca Raton, pp 69–93

Derenzini M, Farabegoli F (1987) Toluidine blue 0 as a Schiff-like reagent for Lowicryl-embedded tissues in light and electron microscopy. Basic Appl Histochem [Suppl] 31:65

Derenzini M, Viron A, Puvion-Dutilleul F (1982) The Feulgen-like osmium ammine reaction as a tool to investigate chromatin structure in thin sections. J Ultrastruct Res 80:133–147

Donovan RM, Goldstein E (1985) A charge coupled device-based image cytophotometry system for quantitative histochemistry and cytochemistry. J Histochem Cytochem 33:551–556

Driel-Kulker AMJ van, Mesker WE, Velzen I van, Tanke HJ, Feichtinger J, Ploem JS (1985) Preparation of monolayers smears from, paraffin-embedded tissue for image cytometry. Cytometry 6:268–272

Driel-Kulker AMJ van, Eysackers MJ, Dessing MTM, Ploem JS (1986) A simple method to select specific tumor areas in paraffin blocks for cytometry using incident fluorescence microscopy. Cytometry 7:601–604

Duijndam WAL, Van Duijn P (1975) The influence of chromatin compactness on the stoichiometry of the Feulgen-Schiff procedure studied in model films. II. Investigations on films containing condensed or swollen chicken erythrocyte nuclei. J Histochem Cytochem 23:891–900

Feulgen R, Rossenbeck H (1924) Mikroskopisch-chemischer Nachweis einer Nukleinsäure von Typus der Thymonukleinsäure und die darauf beruhende selektive Färbung von Zellkernen in mikroskopischen Präparaten. Hoppe-Seylers Z Physiol Chem 135:203–248

Finck H (1958) An electron microscope study of basophile substances of frozen-dried rat liver. J Biophys Biochem Cytol 4:291–299

Gaillard JLJ, Van Duijn P, Schaberg A (1968) Photometric determination of DNA in human chromosomes Exp Cell Res 53: 417–431

Gautier A (1976) Ultrastructural localization of DNA in ultrathin tissue sections. Int Rev Cytol 44:113–191

Gautier A, Schreyer M (1970) Feulgen-like electron stains for tissue sections. In: Favard P (ed) Microscopie electronique 1970, vol 1. Soc Franc Microscopie Electronique, Paris, pp 559–560
Gil JE, Jotz MM (1976) Further observations on the chemistry of pararosaniline-Feulgen staining. Histochemistry 46:147–160
Goelz SE, Hamilton SR, Volgestein B (1985) Purification of DNA from formaldehyde-fixed and paraffin-embedded human tissue. Biochem Biophys Res Commun 130:118–126
Goldstein DJ (1970) Aspects of scanning microdensitometry. I. Stray light (glare). J Microsc 92:1–16
Goldstein DJ (1971) Aspects of scanning microdensitometry. II. Spot size, focus and resolution. J Microsc 93:15–42
Goldstein DJ (1975) Aspects of scanning microdensitometry. III. The monochromator system. J Microsc 105:33–56
Grove GL, Anderton R, Smith JG Jr (1976) Cytophotometric studies of epidermal proliferation in psoriasis and normal skin. J Invest Dermatol 66:236–238
Hall TL, Fu YS (1985) Applications of quantitative microscopy in tumor pathology. Lab Invest 53:5–21
Hedley DW, Friedlander ML, Taylor IW, Rugg CA, Musgrove EA (1983) Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. J Histochem Cytochem 11:1333–1335
Hiddeman W, Schumann J, Andreef M, Barlogie B, Herman CJ, Leif RC, Mayall BH, Murphy RF, Sandberg AA (1984) Convention on nomenclature for DNA cytometry. Cancer Genet Cytogenet 13:181–183
Hillary BB (1939) Use of the Feulgen reaction in cytology. I. Effect of fixatives on the reaction. Bot Gaz 101:276–300
Hörmann H, Grassmann W, Pries G (1958) Über der Mechanismus der Schiffschen Reaktion. Justus Liebigs Ann Chem 616: 125–147
Iwamoto KS, Mizuno T, Ito T, Akiyama M, Takeichi N, Mabuchi K, Seyama T (1996) Feasibility of using decades-old archival tissues in molecular oncology/epidemiology. Am J Pathol 149: 399–406
Kamentsky LA, Melamed MR (1969) Instrumentation for automated examinations of cellular specimens. Proc IEEE 57:2007
Kamentsky LA, Melamed MR, Derman H (1969) Spectrophotometer: new instrument for ultrarapid cell analysis. Science 150: 630
Kasten FH (1958) Additional Schiff-type reagents for use in cytochemistry. Stain Technol 33:39–45
Kasten FH (1960) The chemistry of Schiff's reagent. Int Rev Cytol 10:1–100
Keebler C (1991). Cytopreparatory techniques. In: Bibbo M (ed) Comprehensive cytopathology. Saunders, Philadelphia, pp 881–906
Kiss R, Gasperin P, Verhest A, Pasteels J-L (1992) Modification of tumor ploidy level via the choice of tissue taken as diploid reference in the digital cell image analysis of Feulgen-stained nuclei. Mod Pathol 5:655–660
Kjellstrand PTT (1977a) Control of extraction of deoxyribonucleic acid and apurinic acid by polyethylene glycol in Feulgen hydrolysis. J Histochem Cytochem 25:371–375
Kjellstrand PTT (1977b) Temperature and acid concentration in the search for optimum Feulgen hydrolysis conditions. J Histochem Cytochem 25:129–134
Kompmann M, Paddags I, Sandritter W (1966) Feulgen cytophotometric DNA determinations on human hearts. Arch Pathol 82:303–308
Kraemer PM, Petersen DF, Van Dilla MA (1971) DNA constancy in heteroploidy and stem line theory of tumors. Science 74: 714–717
Lapham LW (1962) Cytologic and cytochemical studies of neuroglia. Part I. A study of the problem of amitosis in reactive protoplasmic astrocytes. Am J Pathol 41:1–21
Lee T-K, Wiley AL Jr, Esinhart JD, Riley RS, Blackburn LD (1993) Variations associated with disaggregation methods in DNA flow cytometry. Anal Quant Cytol Histol 15:195–200
Leeman U, Weiss S, Schmutz E (1971) A centrifugation technique for cytochemical preparations. J Histochem Cytochem 19: 758–760
Lessler MA (1953) The nature and specificity of the Feulgen reaction. Int Rev Cytol 2:231–247
Mairinger T, Gschwendtner A (1996) Comparison of different mathematical algorithms to correct DNA-histograms obtained by measurements on thin liver tissue sections. Anal Cell Pathol 159–171
Marchevsky A, Tolmachoff T, Lee S (1996) Quality assurance issues in DNA image cytometry. Cytometry 26:101–107
Marinozzi V (1966) Fine structure of interphase nuclei. Lo Sperimentale 116: 381–435
Melchiorri C, Bolondi L, Chieco P, Pagnoni M, Gramantieri L, Barbara L (1994) Diagnostic and prognostic value of DNA ploidy and cell nuclearity in ultrasound guided liver biopsies. Cancer 74:1714–1719
Mellin W (1990) Cytophotometry in tumor pathology. A critical review of methods and applications, and some results of DNA analysis. Pathol Res Pract 186:37–62
Mentre P (1972) Coloration sur coupes ultra-fines des acides nucleiques et des certains polysaccharides par le thallium en solution alcoolique. J Microscopie 7:251–270
Mikel UV, Becker RL Jr (1991) A comparative study of quantitative stains for DNA in image cytometry. Anal Quant Cytol Histol 13:253–260
Mikel UV, Fishbein WN, Bahr GF (1985) Some practical considerations in quantitative absorbance microspectrophotometry. Preparation techniques in DNA cytophotometry. Anal Quant Cytol Histol 7:107–118
Miner RW, Kopac MJ (1956) Cancer cytology and cytochemistry. Ann N Y Acad Sci 63:1033
Moyne G (1973) Feulgen-derived techniques for electron microscopical cytochemistry of DNA. J Ultrastruct Res 45:102–123
Moyne G (1974) Mise en evidence de l'ADN par la reaction au Schiff-thallium. J Microscopie 21:205–208
Moyne G (1980) Methods in ultrastructural cytochemistry of the cell nucleus. Prog Histochem Cytochem 13:1–72
National Toxicology Program (1998) Eighth report on carcinogens. US Department of Health and Human Services, Washington DC
Nitsch B, Murken JD, Bruck HJ (1970) Determining Feulgen-DNA of individual chromosomes by fluorescence cytophotometry with incident light. Histochemie 23:254–265
Olins AL, Moyer BA, Kim SH, Allison DP (1989) Synthesis of a more stable osmium ammine electron dense DNA staining. J Histochem Cytochem 37:395–398
Ornstein L (1952) The distributional error in microspectrophotometry. Lab Invest 1:250–265
Oud PS, Zahniser DJ, Garcia GL, Boekel MCG van, Haag DJ, Hermkens HG, Pahlplatz MMM, Vooijs PG, Herman CJ (1984) Pressure-filtration method of transferring cells from polycarbonate filters to glass slides. Anal Quant Cytol 6: 131–138
Overton WR, McCoy JP (1994) Reversing the effect of formalin on the binding of propidium iodide to DNA. Cytometry 16: 351–356
Patau K, Swift H (1953) The DNA-content (Feulgen) of nuclei during mitosis in a root tip of onion. Chromosoma 6:149–169
Pearse AGE (1985) Histochemistry. Theoretical and applied, 4th edn, vol 2. Churchill Livingston, Edinburgh, pp 611–674
Peters D (1966) Electron microscopic studies on the localization of deoxyribonucleic acid inside of DNA viruses. In: Uyeda R (ed) Electron microscopy 1966, vol II. Maruzen, Tokyo, pp 195–196
Peters D, Giese H (1970) Detection of DNA in thin sections. In: Favard P (ed) Microscopie electronique 1970, vol 1, Soc Franc Microscopie Electronique, Paris, pp 557–558
Pollister AW, Ornstein L (1959) The photometric chemical analysis of cells. In: Mellors RC (ed) Analytical cytology, 2nd edn. McGraw-Hill, New York, pp 431–518
Pollister AW, Ris H (1947) Nucleoprotein determinations in cytological preparations. Cold Spring Harbor Symp Quant Biol 12:147–157

Pompella A, Maellaro E, Casini AF, Comporti M (1987) Histochemical detection of lipid-peroxidation in the liver of bromobenzene-poisoned mice. Am J Pathol 129:295–301

Prenna G, Leiva S, Mazzini G (1974) Quantitation of DNA by cytofluorometry of the conventional Feulgen reaction. Histochem J 6:467–489

Puchtler H, Meloan SN, Brewton BR (1975) On the history of basic fuchsin and aldehyde-Schiff reactions from 1862–1935

Puvion-Dutilleul F, Leduc EH, Puvion E (1996)The osmium amine-SO_2 staining method for studying the in situ configuration of viral genomes in ultrathin sections of DNA virus infected cells. Biol Cell 87:133–141

Rasch RW, Rasch EM (1973) Kinetics of hydrolysis during the Feulgen reaction for deoxyribonucleic acid. A re-evaluation. J Histochem Cytochem 21:1053–1065

Roehrl MH, Becker KF, Becker I, Hofler H (1998) Efficiency of single-cell polymerase chain reaction from stained histologic slides and integrity of DNA in archival tissue. Diagn Mol Pathol 6:292–297

Rost FWD (1980) Quantitative histochemistry. In: Pearse AGE (ed) Histochemistry. Theoretical and applied, 4th edn, vol 1. Churchill Livingston, Edinburgh, pp 379–417

Sandritter W (1979) A review of nucleic acid cytophotometry in general pathology. In: Pattison JR, Bitensky L, Chayen J (eds) Quantitative cytochemistry and its applications. Academic Press, London, pp 1–8

Schiff H (1866) Eine neue Reihe organischer Diamine. Justus Liebigs Ann Chem 140:92–137

Schimmelpenning H, Falkmer UG, Hamper K, Seifert G, Auer GU (1990) Variations in Feulgen stainability of epithelial parenchymal cells extracted from paraffin-embedded salivary gland specimens. Cytometry 11:475–480

Schwarz G, Wittekind D (1982) Selected aminoacridines as fluorescent probes in cytochemistry in general and in the detection of cancer cells in particular. Anal Quant Cytol 4:44–54

Stephenson RA, Gay H, Fair WR, Melamed MR (1986) Effect of section thickness on quality of flow cytometric DNA content determinations in paraffin-embedded tissues. Cytometry 7: 41–44

Swift H (1950) The constancy of desoxyribose nucleic acid in plant nuclei. Proc Natl Acad Sci 36:643–654

Swift H (1966) Microphotometry in biological research. J Histochem Cytochem 14:842–852

Takamatsu T, Nakanishi K, Onouchi Z, Fukuda M, Fujita S (1980) Nonspecific (“pseudo-plasmal”) dye-binding in the Feulgen nuclear stain and its blocking by azocarmin G. Histochemistry 66:169–180

Thiery JP (1967) Mise en evidence des polysaccharides sur coupes fines en microscopie electronique. J Microscopie 6:987–1018

Thomson RY, Heagy FC, Hutchison WC, Davidson JN (1953) The deoxyribonucleic acid content in rat cell nucleus and its use in expressing the results of tissue analysis, with particular reference to the composition of liver tissue. Biochem J 53:460–474

Thunnissen FBJM, Ellis IO, Jütting U (1997) Quality assurance in DNA image analysis on diploid cells. Cytometry 27:21–25

Tiersch TR, Chandler RW, Wachtel SS, Elias S (1989) Reference standards for flow cytometry and application in comparative studies of nuclear DNA content. Cytometry 10:706–710

Trujillo TT, Van Dilla MA (1972) Adaptation of the fluorescent Feulgen reaction to cells in suspension for flow microfluorometry. Acta Cytol 16:26–30

Van Dilla MA, Trujillo TT, Mullaney PF, Coulter JR (1969) Cell microfluorometry: a method for rapid fluorescence measurement. Science 163:1213

Van Duijn P (1956) A histochemical specific thionine-SO_2 reagent and its use in a bi-color method for deoxyribonucleic acid and periodic acid Schiff positive substances. J Histochem Cytochem 4:55–63

Vendrely R, Vendrely C (1948) La teneur du noyau cellulaire en acid desoxyribonucléique à traverse le organs, les individus et les especes animales. Experientia 4:434–436

Veneti S, Ioannidou-Mouzaka L, Toufexi H, Xenitides J, Anastasiadis P (1996) Imprint cytology. A rapid, reliable method of diagnosing breast malignancy. Acta Cytol 40:649–652

Wied GL, Bartels PH, Dytch HE, Bibbo M (1983) Rapid DNA evaluation in clinical diagnosis. Acta Cytol 27:33–37

Williams RA, Baak JPA, Meijer GA, Charlton IG (1996) Further testing of the value of a measurement protocol for DNA ploidy studies using image analysis. Comparison of results obtained from cytologic and paraffin section preparations. Anal Quant Cytol Histol 18:345–350

Wohnrath DR, Oyafuso MS, Bortolan J, Hidalgo GS, Filho AL (1995) Washed-biopsy cytology of abdominal lesions. Acta Cytol 39:153–156

Yasuzumi G, Sugihara R (1965) The fine structure of nuclei as revealed by electron microscopy. Exp Cell Res 37:207–229

Zimmer HG (1973) Microphotometry. Mol Biol Biochem Biophys 14:297–328