

REVIEW ARTICLE

Cytochemistry of DNA, RNA and nuclear proteins*

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ABSTRACT

Classical (Feulgen, basophilia and acidophilia reactions and optical anisotropy) and recently developed (critical electrolyte concentration - usual and variant techniques -, tests for apoptosis and AgNOR image analysis) tests for the cytochemistry of DNA, RNA and nuclear proteins are briefly considered in the context of *in vitro* and *in situ* research situations.

The cytochemical reactions for DNA, RNA and nuclear proteins are important tools for cytogenetic, cell biology, and analytical cytopathological studies involving localization and identification of different types of these components as well as their quantification and molecular/supramolecular organization.

There is a recent trend to develop DNA/RNA sequence probes linked to non-specific fluorescent dyes for the identification of specific domains in the substrates (FISH assays). These assays, though endowed with great confidence, are not properly cytochemical but molecular biology procedures, and will not be treated here.

The Feulgen reaction

The Feulgen reaction, a highly specific and stoichiometric method for DNA, permits identification, localization and quantification. It has been responsible for detection of the DNA amounts per haploid chromosome set in different animal and plant species, and the establishment of polyploidy and aneuploidy in particular cell types, and also the finding that DNA replication is mostly restricted to the S phase of the cell cycle.

The chemical principles and the factors affecting the response to the Feulgen reaction as well as details on the analysis of Feulgen hydrolysis curves and Feulgen spectral curves are reviewed elsewhere (Mello and Vidal, 1978; Vidal, 1987). Briefly, the Feulgen reaction comprises two steps: 1) acid hydrolysis, which induces DNA depurination and unmasking of deoxyribose residues capable of reacting as aldehydes; 2) treatment with Schiff's reagent, a leucoderivative of basic fuchsin, which will react with the apurinic acid aldehydes, giving rise to a magenta-stained product (Figure 1a). It is important to use a basic fuchsin as pure as possible.

If the acid hydrolysis is extended beyond the time that corresponds to the maximal depurination, there will be a gradual solubilization of the apurinic acid. This phenomenon is identified as corresponding to the descending branch of a Feulgen hydrolysis curve (Figure 2).

Concomitant with DNA depurination, the acid hydrolysis step promotes RNA solubilization, partial protein denaturation and release of denatured products from chromatin. Consequently, RNA will not affect the results, but the proteins that remain unextracted from chromatin and/or chromosomes may slow down the acid hydrolysis kinetics to different degrees.

Acid hydrolysis is generally performed with HCl. Concentration and temperature of the hydrolytic bath should be chosen by the experimenter, taking into account reproducibility of the reaction and minimi-

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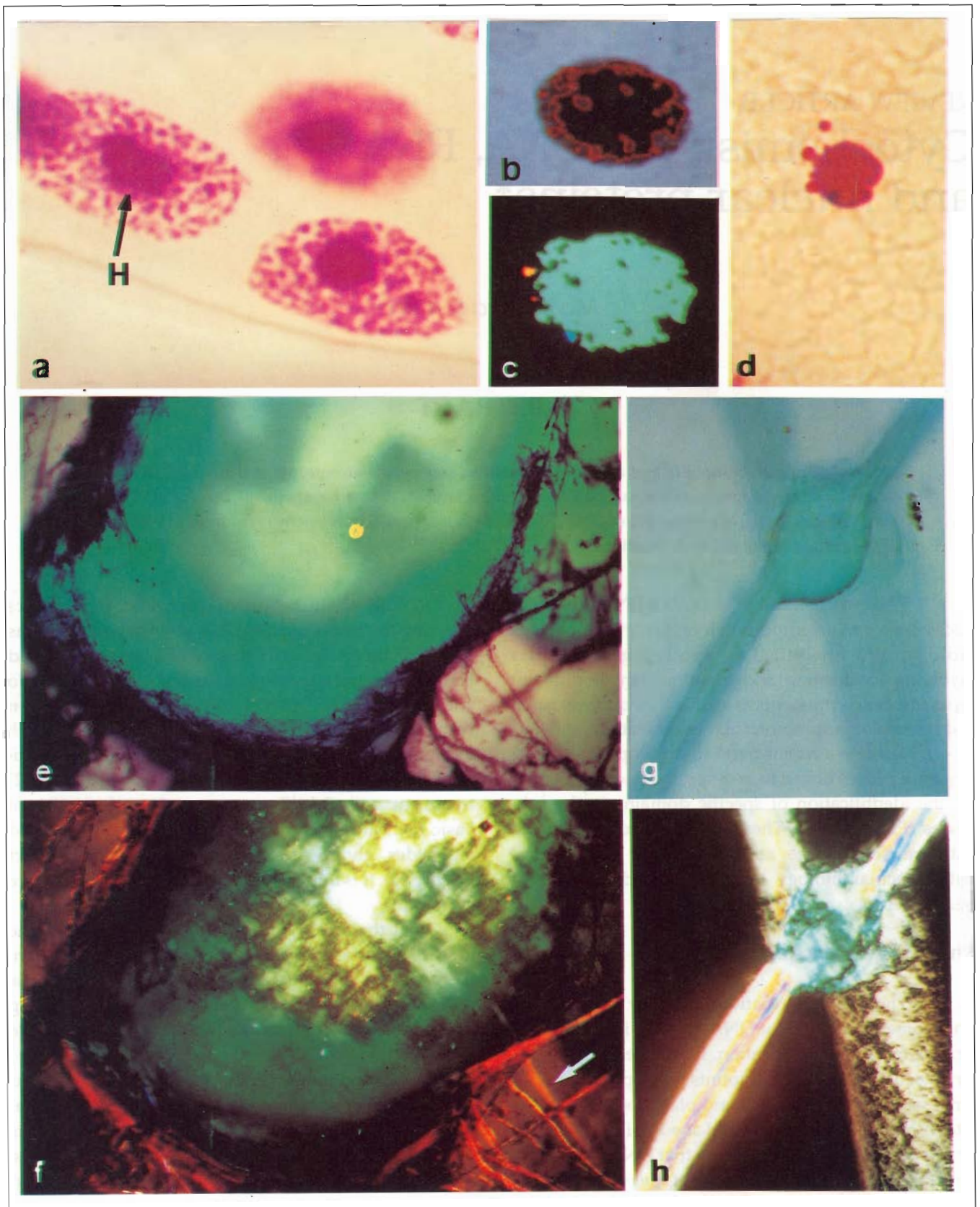


Figure 1 - *a*, Feulgen-stained cell nuclei of *Triatoma infestans*. A heterochromatic body is especially highlighted (H). *b, c*, Segmentation and feature evaluation on video image of a Feulgen-stained nucleus of *ras*-transformed NIH 3T3 cell. Pseudocolored image. Different colors in *c* represent areas separated from each other. *d*, Feulgen-stained apoptotic nucleus of *Triatoma infestans*. *e, f*, TB-stained filaments of pure DNA observed under unpolarized (*e*) and polarized (*f*, arrow) light. Very concentrated DNA masses in the center of the image were not reached by the stain (unstained) or bound a few dye molecules (green color) and show birefringence with a white or silver color. *g, h*, TB-stained filaments of a DNA-protamine complex under unpolarized (*g*) and polarized (*h*) light.

zation of overlap of the DNA depurination and DNA/apurinic acid solubilization steps. Acid hydrolysis with 3.5, 4 or 5 N HCl at room temperature has been generally recommended. The optimal hydrolysis time must always be determined in a preliminary test considering the material for which the Feulgen-DNA content is intended to be evaluated. It is also recommended that the preparations be subjected to a rapid fixation in the ethanol (or methanol): acetic acid (3:1, v/v) mixture classically used in cytogenetics.

Since the patterns of DNA susceptibility to the hydrolytic action are highly affected by proteins involved in chromatin compactness and in association with certain specific DNA types, it is not surprising that different Feulgen hydrolysis kinetics should be revealed in heterochromatin compared to euchromatin, and in chromatin zones containing different proportions of DNA types, defined in terms of their base sequences (reviewed in Mello, 1983).

Different higher-order packing states of the DNA-protein complex in chromatin can be especially envisioned in Feulgen-stained preparations and established and quantified by image analysis procedures which additionally reveal several other of their characteristics (Figure 1b,c) (Vidal, 1984, 1992; Mello, 1989; Mello *et al.*, 1994, 1995). This can be performed, even after restriction endonuclease treatments, which allows the identification, for instance, of methylated DNA-rich zones in interphase chromatin (Mello *et al.*, 1997).

The Feulgen reaction has recently been considered for studies on apoptosis which is also related to the condensed chromatin packing state (Camby *et al.*, 1995) (Figure 1d).

DNA evidence by fluorescence

The Feulgen reaction can also be performed by using very dilute concentrations of basic fuchsin and observed with a fluorescence microscope. Many Feulgen-like reactions using fluorescent dyes or Schiff-like reagents have also been proposed (Mello and Vidal, 1978; Burger *et al.*, 1990).

On the other hand, several dyes including Hoechst 33342, DAPI, chromomycin A3, quinacrin, acridine orange, ethidium bromide and propidium oxide have been tested for DNA stoichiometry in procedures not chemically comparable to a Feulgen reaction. Hoechst stain was considered the most suitable fluorochrome among those used for DNA quantification in fluorescence analysis (Santisteban *et al.*, 1990). Some fluorescent dyes like quinacrin have a much higher affinity for certain DNA bases like AT

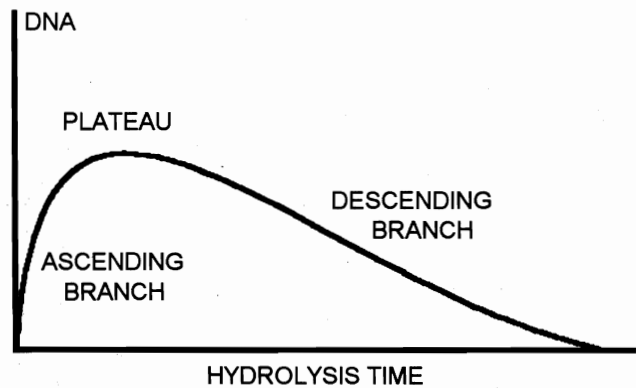


Figure 2 - Feulgen hydrolysis curve. The ascending branch corresponds to DNA depurination while the downslope reveals the apurinic acid depolymerization. Maximal depurination corresponds to the curve plateau.

(Comings, 1978) and acridine orange stains nucleic acids by a mechanism of basophilia and intercalation (Vidal, 1987), as reported below.

Basophilia reactions

Staining with 0.025% toluidine blue (TB) solutions in McIlvaine buffer at pH 4.0 has been indicated for nucleic acid studies since the molecules of this dye bind electrostatically to available DNA and RNA phosphates. RNA can thus be distinguished by detection of changes in basophilia after RNase treatment.

The spectral localization of absorption peaks of the TB-stained chromatin has been associated with the levels of stacking and proximity of the dye molecules bound to the substrate and, consequently, with the availability of substrate binding sites (Lison, 1960; Toepfer, 1970; Vidal, 1987). Naked DNA filaments, with a maximum of free phosphates, and DNA-protamine complexes, with a very small number of free phosphates, for instance, stain violet (metachromasy) and green, respectively (Figure 1e-h). Their absorption peaks, positioned at $\lambda = 540-550$ nm and 625-630 nm, respectively, inform the level of phosphate availability in the DNA (Mello, 1983).

The basophilia reaction in TB-stained preparations also detects differences at the protein level in the chromatin, though indirectly. An example of this approach is revealed in studies of DNA-protein complexes in spermatozoa. Normally shaped spermatozoa in the bull and in some other mammals contain a keratinous protamine tightly associated with the DNA. When subjected to TB staining, these sperm cell nuclei stain green (Figure 3a). However, some normally shaped sperm cells bearing a chemically abnormal DNA-protein complex, in which many DNA phosphates are not bound to

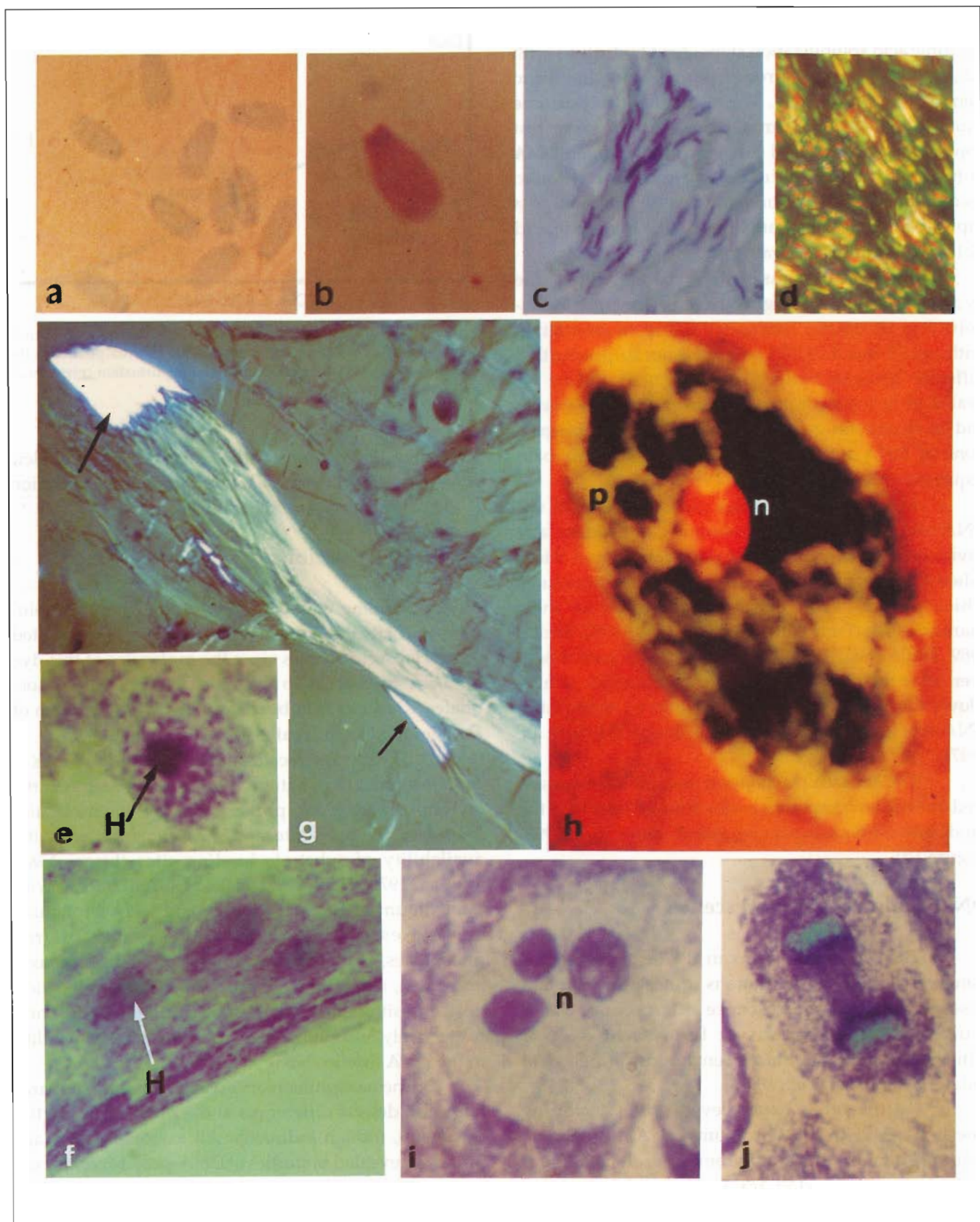


Figure 3 - *a, b*, TB-stained bull spermatozoa. Normal (*a*) and abnormal (*b*) DNA-protein complexes are revealed. *c, d*, TB-stained honey bee spermatozoa under unpolarized (*c*) and polarized (*d*) light. *e, f*, Response of *Triatoma infestans* heterochromatin (H) to TB staining preceded by treatment with protamine (*f*); *e*, control. *g*, TB-stained *T. infestans* spermatozoa exhibiting a negative birefringence typically due to DNA (arrow). *h*, Acridine orange-stained *Megazelia* cell. Red fluorescence is seen in the nucleolus (n) and cytoplasm while a green fluorescence is evident in the polytene chromosomes (p). *i, j*, RNA metachromasy revealed by the CEC method in interphase (*i*) and mitotic (*j*) MCF-10A cells.

the protein, exhibit nuclear metachromasy (Figure 3b) (Mello, 1982; Beletti and Mello, 1996). This sperm cell abnormality is 12 times more frequent in subfertile bulls (Mello, 1982). On the other hand, the sperm cell nuclei of the honey bee, which contain an H1 histone-like protein associated with DNA, stain violet (Figure 3c), as do most somatic cell nuclei (Mello and Vidal, 1973; Mello and Falco, 1996).

Since protamine molecules are known to bind only to double-stranded nucleic acids, somatic interphase chromatin and polytene chromosomes subjected to TB staining and then treated experimentally with protamine will reveal RNA-rich areas and puffs stained metachromatically, while areas devoid of RNA (heterochromatin, for instance) will appear stained in green (Vidal, 1979; Mello, 1985) (Figure 3e,f).

Acridine orange imparts fluorescent emissions of different colors to DNA and RNA. If bound electrostatically to the nucleic acid phosphates (RNA or single-stranded DNA) this dye will produce a red fluorescence, but if intercalated within the nucleic acid double-helix (mostly DNA) it will generate a green fluorescent color (Vidal, 1987) (Figure 3h).

The acetic ethanol fixation is adequate for studies of basophilia, but some controls fixed in buffered formalin or any other aldehyde are also required when there is interest in the participation of nuclear proteins in the generation of specific staining properties.

Optical anisotropy

TB staining followed by examination of the preparations under polarized light reveals some characteristics of the DNA-protein complex type, especially in *in vitro* preparations and in sperm cell nuclei. Pure DNA filaments under unstained conditions exhibit birefringence with a silver interference color, due to the stereoarrangement of the DNA bases overlaid on each other (Figure 1f). If stained with TB, this DNA will exhibit metachromasy (violet color, unpolarized light) and a birefringence with yellow or green interference colors (polarized light), due to the stereoarrangement of the DNA bases, plus that of the dye molecules stacked around the DNA helix (Figure 1f). DNA linked to somatic histones or a lysine-rich sperm nuclear protein after TB staining will also display birefringence characteristics like those of pure DNA (Figure 3d). DNA linked to arginine-rich proteins like protamines *in vitro* or in spermatozoa, on the other hand, will show intensification of the birefringence due to DNA only (silver interference color) (Figures 1h and 3g), since very few TB molecules are attached to the nucleic acid because the majority of its phosphate groups are linked

to protein amino groups (Mello and Vidal, 1973; Vidal and Mello, 1989).

Critical electrolyte concentration

DNA-protein complexes *in vitro* and *in situ*, if stained with TB solutions containing increasing concentrations of Mg^{2+} or Ca^{2+} , will show metachromasy abolishment at a certain concentration in molarity of the inorganic cation. This has been named critical electrolyte concentration (CEC) phenomenon and is applicable to studies of DNA- and RNA-protein complexes in association with different states of chromatin supraorganization (Vidal and Mello, 1989; Mello *et al.*, 1993; Mello and Falco, 1996).

The competition of TB and Mg^{2+} for the substrate dye binding sites has been found to differ, when comparing heterochromatin with euchromatin, spermatozoal nuclei among different animal species, DNA and RNA puffs with heterochromatin and unpuffed polytene chromosome bands, and the same nucleus type under different physiological or developmental conditions (Mello and Vidal, 1989; Monteiro, 1993; Mello and Falco, 1996).

Based on the fact that the CEC point for RNA is always higher than that for DNA, a variant of the CEC method has been proposed in which Mg^{2+} treatment is not simultaneous but follows TB staining (Mello *et al.*, 1993). Under these conditions, while DNA stains green, RNA remains stained metachromatically (Figure 3i,j), allowing special discrimination of rRNA, localization and evidence of suprastructural details of the nucleolus and follow-up of RNA during cell division (Mello *et al.*, 1993; Mello, 1995). Additionally, this method is being proposed as a simple and useful tool for rapid identification and study of apoptotic cells, highlighting chromatin deeply stained in green (DNA) and presence of RNA in the apoptotic bodies stained in violet (Figure 4a,b) (Vidal *et al.*, 1996). If accompanied by an immunocytochemical assay for DNA 3' end-labeling, a marker for apoptosis (Wijsman *et al.*, 1993) (Figure 4c), the CEC assay is quite precise.

Acid dye binding

Anionic dyes can be used for the cytochemical demonstration of nuclear proteins through electrostatic binding of the dye anions to the available cationic groups of proteins. DNA thus must be extracted with TCA to avoid competition for binding sites. Fixation in buffered formalin or another aldehyde is required.

For demonstration of total nuclear proteins, dyes like naphthol yellow S or fast green FCF at pH 2.7

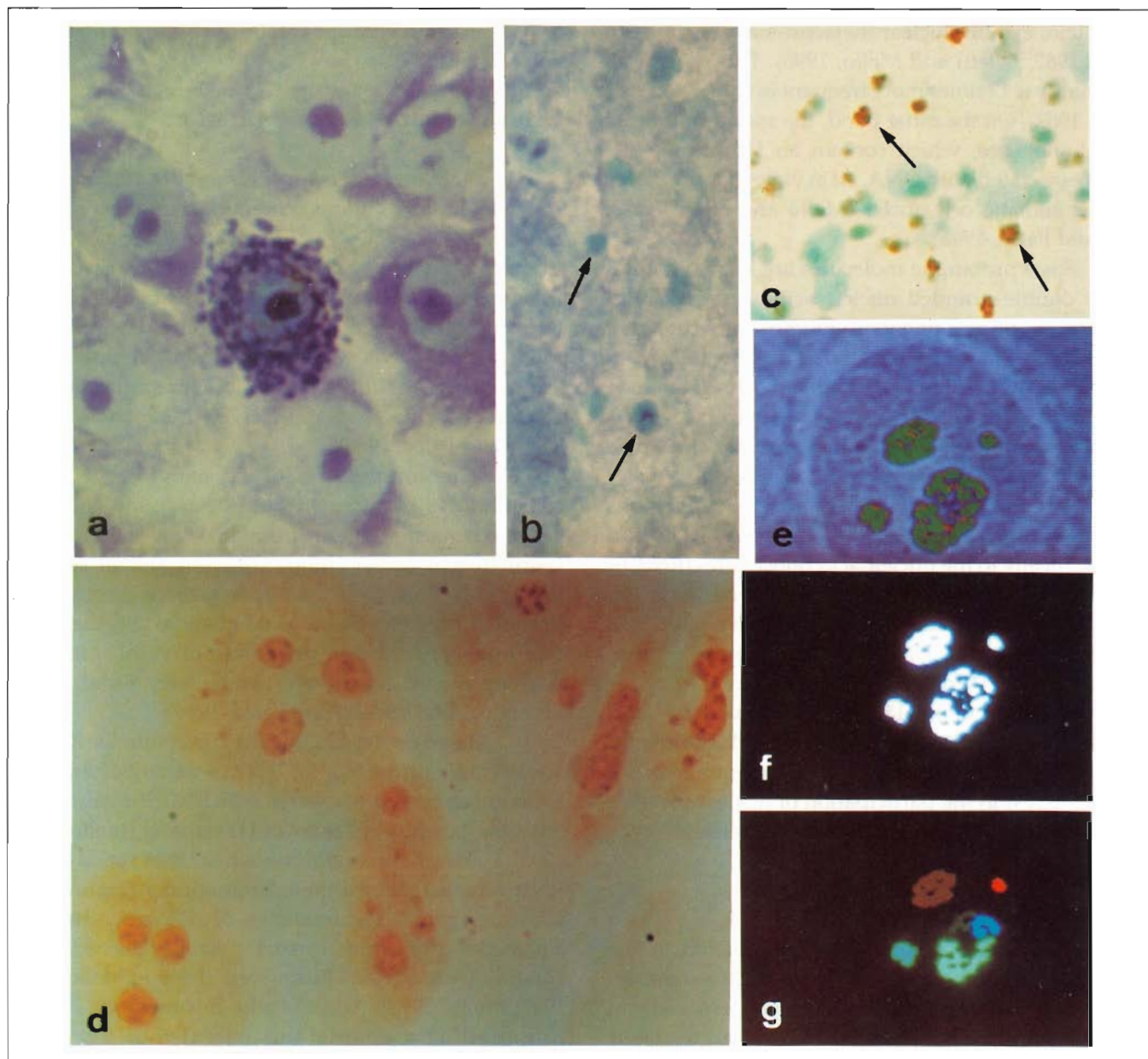


Figure 4 - *a-c*, Apoptosis revealed by the CEC method (*a,b*) and the ApopTag™ assay (*c*) in MCF-10F (*a*) and V79 (*b,c*) cells. Arrows indicate apoptotic nuclei. *d-g*, Ag-NOR staining in MCF-10A cells. Video images show different colors representing positive areas separated from each other.

have been proposed (reviewed in Vidal, 1987). If histones are removed from the cell nuclei by treatment with a NaCl, HCl or H₂SO₄ solution prior to staining, only nonhistone proteins will be demonstrated.

Staining with fast green FCF at pH 8.1 will reveal only histones, on account of their high isoelectric points. Variation in the conformational states of the histones stained with fast green has been reported to affect the amount of dye uptake (Tas *et al.*, 1980). However, the profile of histone extraction kinetics in cells differing in physiological states and stained with fast green may uncover differences in histone conformation associated with nuclear functions (Mello and Cordeiro, 1985).

Treatment with fast green at pH 8.1 preceded by deamination will not stain lysine-rich histones, being useful for distinguishing spermatozoa bearing an H1 histone from those containing an arginine-rich nuclear basic protein (Bloch and Hew, 1960; Bols and Kasinsky, 1972; Taboga *et al.*, 1996).

Ag-NOR staining response

The Ag-NOR staining method and its variants stain associated acidic nuclear proteins at the rDNA sites with silver. Not only NORs in chromosomes, but also components of interphase nucleoli appear stained (Ploton *et al.*, 1987). In fact, it has been suggested that

the technique highlights the presence and amount of the phosphoprotein C23 (nucleolin), but other proteins, including factors necessary for rDNA transcription and early rRNA processing, are reported to contribute to the Ag-NOR staining response (Schwarzacher and Wachtler, 1993).

Many Ag-NOR positive nucleolar proteins adhere to the NORs during mitosis, being responsible for the silver staining of these regions in mitotic chromosomes, and consequently, are of importance in cytogenetics. This Ag-NOR-positive material is regarded as a remnant of the nonchromosomal part of the nucleolus (Schwarzacher and Wachtler, 1993).

The amount of protein which stains with the Ag-NOR technique may indicate the state of activity of the cell as a function of ribosomic synthesis. It is conceivable that the amount of silver-positive material is related to the rate of cell proliferation. The method has even been proposed and used for distinguishing tumor and normal cells (Ploton *et al.*, 1987; Crocker and Egan, 1988; Derenzini *et al.*, 1989; Lesty *et al.*, 1992 - among hundreds of citations). However, the demand for ribosomes is not necessarily related to rapid cell proliferation (ex.: nerve cells, interferon-stimulated monocytes, and polyploid liver cells) (Schwarzacher and Wachtler, 1993; Vidal *et al.*, 1994).

The cytometric characteristics of the Ag-NOR-positive response have recently been studied with image analysis (Lesty *et al.*, 1992; Vidal *et al.*, 1994) (Figure 4d-g) to better distinguish changes associated with normal cell functions, vs. cellular pathological states.

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RESUMO

São brevemente considerados nesta apresentação testes citoquímicos tradicionais (Feulgen, basofilia, acidofilia e anisotropia óptica) e os recentemente desenvolvidos (concentração crítica de eletrólitos - técnica usual e variante; testes para apoptose e análise de imagem de AgNOR) para DNA, RNA e proteínas nucleares, dentro de um contexto de aplicação a pesquisas voltadas a situações *in vitro* e *in situ*.

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