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Original Article

Fluorescence Image Cytometry of Nuclear DNA Content Versus Chromatin Pattern: A Comparative Study of Ten Fluorochromes[1]

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This study is intended to be the first step of an in situ exploration of the intranuclear DNA distribution by image cytometry (SAMBA) with several fluorochromes. The nuclear DNA content and the chromatin pattern, revealed by ten fluorochromes (HO, DAPI, MA, CMA3, OM, QM, AO, EB, PI, and 7-AMD), were analyzed on mouse hepatocytes fixed by the Boehm–Sprenger procedure optimal for preserving the chromatin pattern. The question was whether fluorochromes specific to DNA make it possible to accurately quantitate the total nuclear DNA content when the chromatin pattern is preserved. Only HO and MA were found to provide satisfactory quantitation of nuclear DNA content, as assumed by both a small CV and a 4c to 2c ratio equal to 2. PI, EB, 7-AMD, and OM provided higher CV values, although the 4c to 2c ratio was still equal to 2. QM, AO, CMA3, and DAPI provided non-reproducible and non-stoichiometric nuclear DNA content measurements under the fixation conditions used. The intranuclear and the internuclear SD of the fluorescence intensities describing the fluorescence pattern of the 2c hepatocytes proved to vary according to both the base-pair specificity and the binding mode of the fluorochromes. The results reported here argue in favor of an external binding of 7-AMD to DNA and an increased quantum yield of QM when bound to AT-rich DNA. For PI, EB, 7-AMD, and OM, the measured DNA content increased with the fluorescence distribution heterogeneity. This correlation was not observed with other fluorochromes and is suggested to result from decreased fluorochrome accessibility to DNA when the chromatin is condensed. This study demonstrates that under conditions that preserve chromatin organization, only HO for AT-rich DNA and MA for GC-rich DNA can be used, alone or in combination, to measure nuclear DNA content. With other fluorochromes, either the measured DNA content or the chromatin pattern is assessed in suboptimal conditions when fluorescent image cytometry is used. (*J Histochem Cytochem 40:1789–1797, 1992*)

KEY WORDS: Fluorochromes; Chromatin pattern; DNA content; Fluorescent image cytometry; SAMBA.

Introduction

Understanding the mechanisms involved in gene expression is one of the most challenging tasks in cell biology. There are two approaches to this problem: (a) the molecular approach, which involves homogenizing the cell and breaking down the supramolecular organization, and (b) the in situ methods, which do not destroy cell organization but detect and measure the cell components within their functional environment. This approach avoids artifacts due to spurious contacts between molecules and puts the emphasis on cell-to-cell differences, since analyses are made at the individual cell level.

In situ chromatin organization has been studied by a number of authors at various levels of spatial resolution, including flow cytometry (FCM), microspectrophotometry, image cytometry (ICM), and electron microscopy. The components of the chromatin were detected on the basis of either self-absorption or after staining with different DNA-specific dyes. When fluorescent reporter molecules are used, the measurements were either direct or delayed (time-resolved fluorescence imaging). There is strong evidence from some of these studies that chromatin shows different patterns according to the cell type (Gauvain et al., 1987; Cowden and Curtis, 1981), the state of cell proliferation (Stokke et al., 1988; Rabinovich et al., 1986; Moser et al., 1981), the state of cell differentiation (Giroud et al., 1988; Darzynkiewicz et al., 1984), the set of transcriptionally active genes (Bottone et al., 1989; Bottiroli et al., 1984), the effects of drugs (Block et al., 1987), and neoplasia (Brugal et al., 1986; Oud et al., 1987).

However evocative these studies may be, they failed to produce compelling evidence that organization of particular DNA regions

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is unequivocally dependent on gene expression. This lack of evidence results from the basic limitations inherent in both the stain and measurement methods used until now. Although FCM and microspectrophotometry are highly accurate methods for measuring cellular DNA content and the overall chromatin condensation (Block et al., 1987; Mazzini et al., 1983; Cowden and Curtis, 1981; Moser et al., 1981), they cannot provide meaningful topographical data on individual cells. Only image cytometry can extract the topographical parameters required to study the arrangement of chromatin in situ. The accuracy of the DNA measurements is dependent on the stoichiometry of dye–DNA complex, which, in turn, is influenced by physicochemical (dye/DNA concentration ratio, DNA binding modes and their affinity, ionic strength) (Latt and Langlois, 1990) and biochemical (chromatin supramolecular arrangement) factors (Stokke and Steen, 1987; Larsen et al., 1986; Rabinovitch et al., 1986; Darzynkiewicz et al., 1984). Many fluorescent dyes are assumed to be specific for and bind stoichiometrically to DNA on the basis of FCM studies, given adequate preparation techniques (Taylor and Milthorpe, 1980; Crissman et al., 1978; Ardnt-Jovin and Jovin, 1977; Krishan, 1975). Although these fluorochromes can be used for both flow and image cytometry, no quantitative study of the chromatin pattern obtained with these fluorochromes has been yet made using image cytometry. It was the purpose of this study to determine which of the most commonly used fluorochromes (HO, Hoechst 33342; DAPI, 4'-6-diamidino-2-phenylindole; MA, mithramycin; CMA3, chromomycin A3; OM, olivomycin; QM, quinacrine mustard; AO, acridine orange; PI, propidium iodide; EB, ethidium bromide; and 7-AMD, 7-amino actinomycin D) permits accurate nuclear DNA content measurement under conditions known to preserve chromatin pattern.

Materials and Methods

Biological Material. Mouse hepatocytes were obtained from three mice, 1, 2.5, and 21 months old, sacrificed by cervical dislocation. Liver imprints were made directly on microscopic slides from fresh tissue according to the technique of Shima and Sugahara (1976) and were fixed immediately.

Fixation. All the cell samples were fixed in Boehm–Sprenger fixative (methanol 80%, formaldehyde 15%, acetic acid 5%) for 10 min at room temperature, dehydrated in successive ethanol baths (70, 95, and 100°), and air-dried for storage. Cell samples were later rehydrated just before staining.

Fluorescent Staining. Ten fluorochromes were tested: HO, DAPI, MA, CMA3, OM, QM, AO, PI, EB, and 7-AMD. They were all obtained from Sigma Chemicals (Chimie, France). Various staining procedures were carried out for each of the fluorochromes, either by applying techniques reported in the literature directly or by introducing modifications, mainly in fluorochrome concentrations and buffers, to take into account the fixation procedure used. The staining procedures given in Table 1 are those that resulted in both labeling specificity and the most reproducible DNA content measurements. The staining procedures were carried out either directly after rehydration of the cell samples (HO, DAPI, MA, CMA3, OM, and 7-AMD) or after RNAse treatment (QM, AO, PI, and EB). For RNAse treatment, the cells were incubated between the slide and coverslip with 300 µl of enzyme solution [500 Kunitz units/ml of RNAse A (Sigma) in distilled water] for 90 min at 37°C. The enzymatic action was arrested by washing twice in PBS.

A DNAse pre-treatment was carried out to verify staining specificity. The cells were incubated between slide and coverslip with 300 µl of enzyme solution [1000 Kunitz units/ml of DNAse I (Sigma) in Tris-HCl buffer, pH 7.5, 100 mM $MgCl_2$] for 90 min at 37°C. The enzymatic action was arrested by washing twice in PBS.

After staining, the slides were rinsed twice for 5 min in buffered solutions to remove background fluorescence, except in the case of EB, for which slides were mounted in glycerol with fluorochrome solution because the rinsing step led to weak fluorescence intensity. Finally, the slides were mounted in glycerol, 0.23% DABCO (Sigma), 0.02% NaN_3, and immediately analyzed.

Fluorescent Quantitative Analysis. Image cytometry (ICM) was carried out using a SAMBA (System for Analytical Microscopy in Biomedical Applications; Alcatel, Meylan, France and Dynatech Laboratories, Springfield, MO). The system consists of an epi-illumination microscope with a 50-W mercury arc lamp (Zeiss Axiophot) connected to a SIT camera (4036; Lhesa Electronique, Cergy–Pontoise, France), a preprocessor (Matrox Mip), an image analysis processor (TITN Co), and a host computer (Victor 386). The hardware and software packages of the system have already been described elsewhere (Giroud, 1986; Brugal, 1984).

Each fluorescent image was obtained through a ×40, NA 0.75 objective and ×2.0 projective lenses and digitized into a 512 × 512 image frame onto 256 levels. The excitation and light collection wavelengths used for each fluorochrome are listed in Table 2.

The image analysis set up involved:

- The acquisition of a homogeneous fluorescent field consisting of a DNA–gelatin spot stained with each fluorochrome tested. This procedure provided a reference digital image which made it possible to correct for the overall spatial inhomogeneity of the analysis system (illumination, optics, camera shading) (Camus et al., 1990).
- The acquisition of an image from the background of the cell preparation to correct for possible nonspecific fluorescence outside the nuclei.
- The interactive adjustment of the thresholds for object selection and segmentation, respectively.

ICM was carried out on 300–400 cell nuclei per slide according to the following procedure:

- At low resolution the nuclei were automatically located and selected on the basis of three thresholds (fluorescence intensity, area, and form factor).
- At high resolution the digital image was corrected, pixel by pixel, for fluorescent background and heterogeneity. Among the parameters calculated by the analyzer, only the following were selected: IFN, integrated fluorescence of a nucleus, representing the measured DNA content; and SDN, standard deviation of intranuclear fluorescence intensities, representing the chromatin pattern.

The nuclear parameters were processed to derive:

- Histograms of the integrated nuclear fluorescence of the hepatocytes cell population. These DNA histograms were standardized by setting the mean IFN value of the diploid mode at 2000 arbitrary units then assumed to represent the 2c DNA content.
- SD_{IFN}, standard deviation of IFN for the 2c cell population.
- CV_{IFN}, coefficient of variation of IFN for the 2c cell population, calculated as the ratio between SD_{IFN} and the mean IFN value.
- M_{SDN}, mean of the SDN for the 2c cell population, representing the average fluorescence pattern of the 2c cell population.
- SD_{SDN}, standard deviation of the SDN for the 2c cell population, representing the internuclear variability of fluorescence patterns.
- CV_{SDN}, coefficient of variation of SDN for the 2c cell population, calculated as the ratio between SD_{SDN} and M_{SDN}. It represents the variablility of the fluorescence patterns relative to the average pattern.

Table 1. *Staining conditions used for the fluorochromes*

Fluorochrome (reference)	Concentration (μg/ml)[a]	Buffer[d]	Time (min)	Temperature	Rinsing
HO (Shapiro, 1981)	0.51	PBS, pH 7.0	30	37°C	PBS buffer
DAPI (Hamada and Fujita, 1983)	0.05	10 mM Trizma base, 10 mM EDTA, 100 mM NaCl, 10 mM cysteine	20	4°C	Trizma
MI (Leemann and Ruch, 1982)	30	McIlvaine, pH 7.0, 10 mM $MgCl_2$	30	Room	McIlvaine
CMA3	100	McIlvaine, pH 7.0, 10 mM $MgCl_2$	30	Room	McIlvaine
OM	100	McIlvaine, pH 7.0, 10 mM $MgCl_2$	30	Room	McIlvaine
QM[c]	5	McIlvaine, pH 7.0, 10 mM $MgCl_2$	30	Room	McIlvaine
AO[b,c] (Darzynkiewicz, 1979)		Hydrolysis: 0.1 N HCl, 0.1 mM EDTA, 0.15 M NaCl	1	0°C	
	9.25	Staining: McIlvaine, pH 6.0, 1 mM EDTA, 0.15 M NaCl	20	Room	McIlvaine
PI[c] (Fried et al., 1976)	10	Na citrate (0.3 mM)	60	Room	Na citrate
EB[c]	1	Tris-HCl, pH 8.0, 1 v, Glycerol, 9v, DABCO, 20 mM, NaN_3, 3 mM	60	Room	None
7-AMD (Zelenin et al., 1984)	12.70	McIlvaine, pH 7.0	20	Room	McIlvaine

[a] Concentration.
[b] Staining procedure after hydrolysis.
[c] Staining procedure after RNAse pre-incubation.
[d] Buffer solutions: Trizma base [Tris(hydroxymethyl)aminomethane] was purchased from Sigma; McIlvaine, pH 7.0, 2 ml of a mix of 0.1 M citric acid (1 v): 0.2 M disodium hydrogen phosphate (4 v), completed to 50 ml with distilled water.

SI, stoichiometric index, calculated as the ratio between the mean IFN value of the cells in the 4c mode and the mean IFN value of the cells in the 2c mode. It represents the linearity of the measured IFN.

Results

DNA Staining Distributions

Figure 1 shows the variety of fluorescence chromatin patterns observed on the hepatocytes stained with the different fluorochromes. The various fluorochromes obviously stained the DNA differently, as revealed by these patterns. The AT-specific HO and DAPI fluorochromes revealed a few bright large spots within a fairly homogeneous background. An obviously different pattern was obtained with the GC-specific MA, CMA3, OM, and 7-AMD, and was characterized by a few dark large spots highlighted by a fluorescent ring. With respect to size and number, the bright spots stained by the AT-specific fluorochromes were similar to those dark spots obtained with the GC-specific fluorochromes. Nevertheless, slight differences of the nuclear patterns were observed from fluorochrome to fluorochrome within a category having the same BP specificity. Finally, the PI, EB, QM, and AO, which have no BP specificity, revealed a chromatin network denser than when stained with any other fluorochromes and characterized by bright regions of various sizes, shapes, and fluorescence intensities.

Figure 2 shows a comparison of the fluorochromes according

Table 2. *Excitation and light collection wavelengths (nm) for fluorescent analysis*[a]

Fluorochromes	Excitation	Dichroic	Light collection
HO, DAPI	BP 365 ± 12	FT 395	LP 397
MA, CMA3, OM	G 436	FT 510	LP 520
QM, AO	BP 450–490	FT 510	LP 520
PI, EB, 7-AMD	BP 546 ± 12	FT 580	LP 590

[a] BP, bandpass filter; G, solid glass filter; FT, chromatic beam splitter; LP, long-wave pass filter.

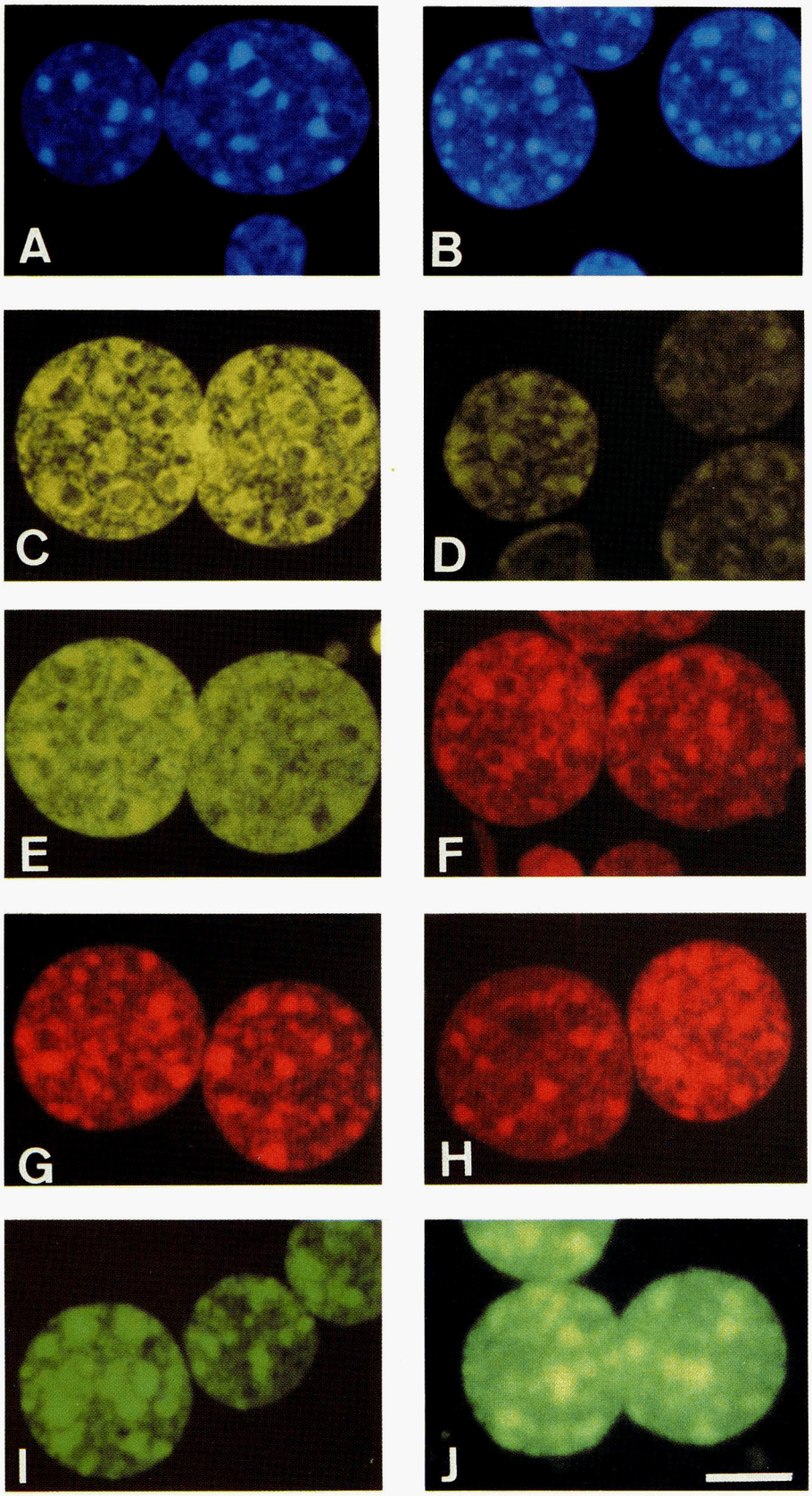

Figure 1. Chromatin patterns obtained with ten different fluorochromes tested on mouse hepatocytes. From top to bottom and left to right: the AT-specific fluorochromes (**A**) HO, (**B**) DAPI, the GC-specific fluorochromes (**C**) MA, (**D**) CMA3, (**E**) OM, (**F**) 7-AMD, and the fluorochromes binding without BP specificity (**G**) PI, (**H**) EB, (**I**) AO, (**J**) QM. Bar = μm.

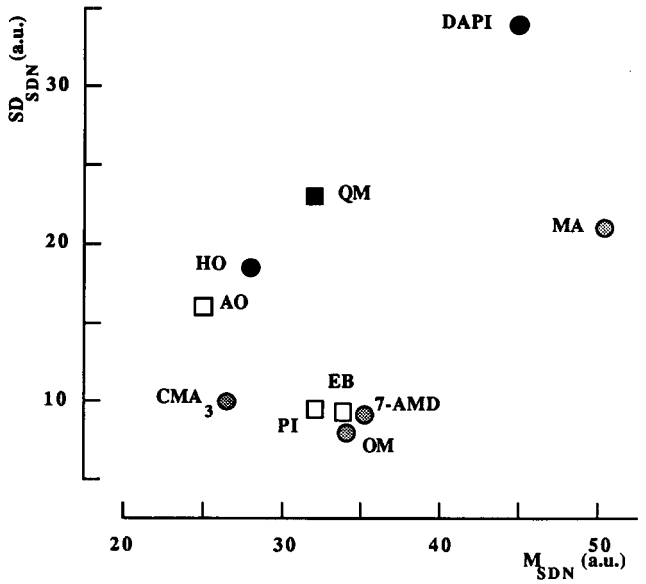

Figure 2. Scattergram of the ten fluorochromes plotted according to the M_{SDN} and the SD_{SDN} (arbitrary units) for the 2c hepatocytes. The parameter M_{SND} is the population mean value of the heterogeneity of the intranuclear distribution of the fluorescence intensities, and the parameter SD_{SDN} is the internuclear variability of this heterogeneity. Circles represent the fluorochromes externally binding to DNA; squares represent the fluorochromes with an intercalating mode of binding. Symbols in black represent the AT-specific fluorochromes (for their binding to DNA, HO, and DAPI, or their fluorescence enhancement, QM); spotted symbols represent the GC-specific fluorochromes; symbols in white represent fluorochromes without BP specifity. a.u., arbitrary units.

to SD_{SDN} plotted against the M_{SDN} of the 2c cell subpopulation of hepatocytes. This figure shows that both the intranuclear (M_{SDN}) and internuclear (SD_{SDN}) variability of the fluorescence intensities clearly depend on the fluorochrome. Moreover, Figure 2 shows a clustering of the fluorochromes according to both their base-pair specificity and binding mode. The AO, PI, and EB, which intercalate into DNA without BP specificity had a relatively small intra- and internuclear variability. The CMA3, OM, MA, and 7-AMD, which are GC specific and externally bound to DNA (see Discussion for 7-AMD mode of binding) had the smallest internuclear variability but very different internuclear variability from one another. The HO and DAPI, which are AT specific, as well as QM (whose fluorescence quantum yield is enhanced when bound to AT) showed high internuclear variability and very different intranuclear variability. These results demonstrated that different fluorochromes considered to be specific for DNA do not reveal the same chromatin regions in situ.

Quantitative Fluorescence Analysis

It must be remembered that normal liver cell populations contain only diploid 2c and polyploid 4c and 8c cells but no S cell with intermediate DNA content. Moreover, since the percentages of 2c, 4c, and possibly 8c cells are known to vary from mouse to mouse and also as a function of the age of the mice (Shima and Sugahara, 1976), the number of cells in every DNA content class has no meaning and therefore did not depend on the fluorochrome used. Only the 2c and 4c cell subpopulations were taken into account, since the percentage of 8c cells was negligible in most cases.

Figure 3 shows the distributions of hepatocyte populations according to the IFN obtained with the various fluorochromes studied. The quantitative analysis of DNA generated two types of histograms. One type showed a bimodal distribution of IFN with one 2c mode and one 4c mode and was obtained with seven fluorochromes: HO, MA, 7-AMD, OM, EB, PI, and QM. The SI averaged 2.00 ± 0.14 among these fluorochromes. The CV_{IFN} values calculated from all cells belonging to the 2c peak ranged from a minimum of 7% for HO to a maximum of 16% for QM. A second type was a continuous distribution of IFN from the 2c to 4c values observed for AO, CMA3, and DAPI. The SI was therefore not calculated. These results show that, given one fixation procedure, the different fluorochromes provided DNA histograms

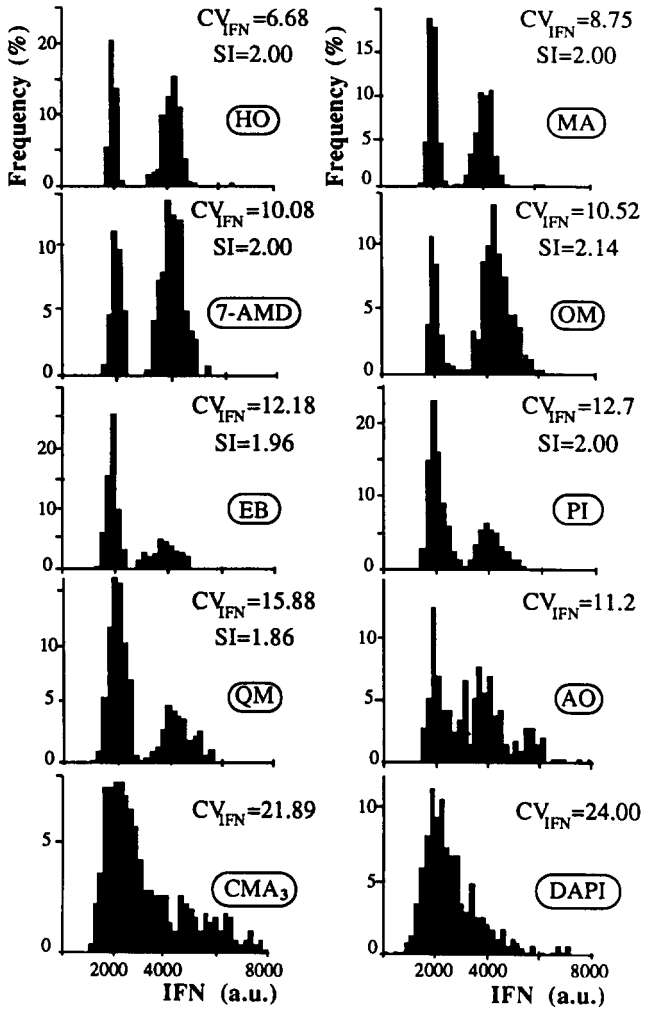

Figure 3. Nuclear integrated fluorescence (IFN) histograms of mouse hepatocytes stained with HO, MA, 7-AMD, OM, EB, PI, QM, AO, CMA3, and DAPI. The 2c peak (left peak) was rescaled at 2000 arbitrary units for each fluorochrome. The coefficient of variation (CV_{IFN}) of the 2c mode is given for each histogram as well as the stoichiometric index (SI), calculated as the ratio between the mean IFN of 4c and 2c cell subpopulations. a.u., arbitrary units.

different in terms of CV but very similar in terms of SI when the 2c and 4c peaks are clearly separated. In the conditions of these experiments, the AO, CMA3, and DAPI were obviously not suitable for reproducible DNA measurements.

Relationship Between DNA Measurement and Fluorescence Pattern

The possible correlation between the intranuclear fluorescence distribution (chromatin pattern) assessed by SDN and IFN was analyzed among the 2c cells at both the individual cell and the cell population levels.

At the individual cell level, three groups of fluorochromes could be clearly separated according to SDN and IFN, as illustrated in Figure 4. In the first group, comprised of HO (Figure 4a) and MA, the DNA content as measured by IFN did not vary extensively from cell to cell (CV_{IFN} between 7% and 9%). In addition, the correlation between the measured DNA content (IFN) and the intranuclear fluorescence distribution (SDN) was not significant (ρ = 3% for HO and 13% for MA). In the second group, comprised of QM (Figure 4b), CMA3, AO, and DAPI, the IFN varied widely from cell to cell (CV_{IFN} from 11% to 24%). Despite this variation of IFN, the correlation between the measured DNA content (IFN) and the intranuclear fluorescence distribution (SDN) was not significant (ρ = −38% for QM, −20% for AO, and −25% for CMA3). In the third group, comprising EB (Figure 4b), PI, 7-AMD, and OM, the IFN increased with SDN although this parameter did not vary extensively among the 2c cell population. The correlation was significant ($p<0.005$) (ρ = 44% for OM, 55% for 7-AMD, 56% for PI, and 72% for EB). These results show that the DNA content measured from these four last fluorochromes, given the experimental conditions, increased with the heterogeneity of the intranuclear fluorescence distribution.

At the population level, the intranuclear fluorescence distribution (SDN) and the measured DNA content (IFN) were compared on the basis of their respective CV. The results are presented in Figure 5 and make it possible to identify the same three groups of fluorochromes already defined at the individual cell level. In the first group, comprising HO and MA, the CV_{SDN} was in the range of 41–66%, with a CV_{IFN} among the smallest values. In the second group, comprising QM, AO, CMA3, and DAPI, the CV_{SDN} was in the range of 40–76%, with a CV_{IFN} among the highest values. In the third group, comprising OM, 7-AMD, PI, and EB, both the CV_{SDN} (24–29%) and the CV_{IFN} (10–12%) were small. These results obviously show that the internuclear variability of the fluorescent patterns (CV_{SDN}) is not directly related to the variability of the measured DNA content (CV_{IFN}).

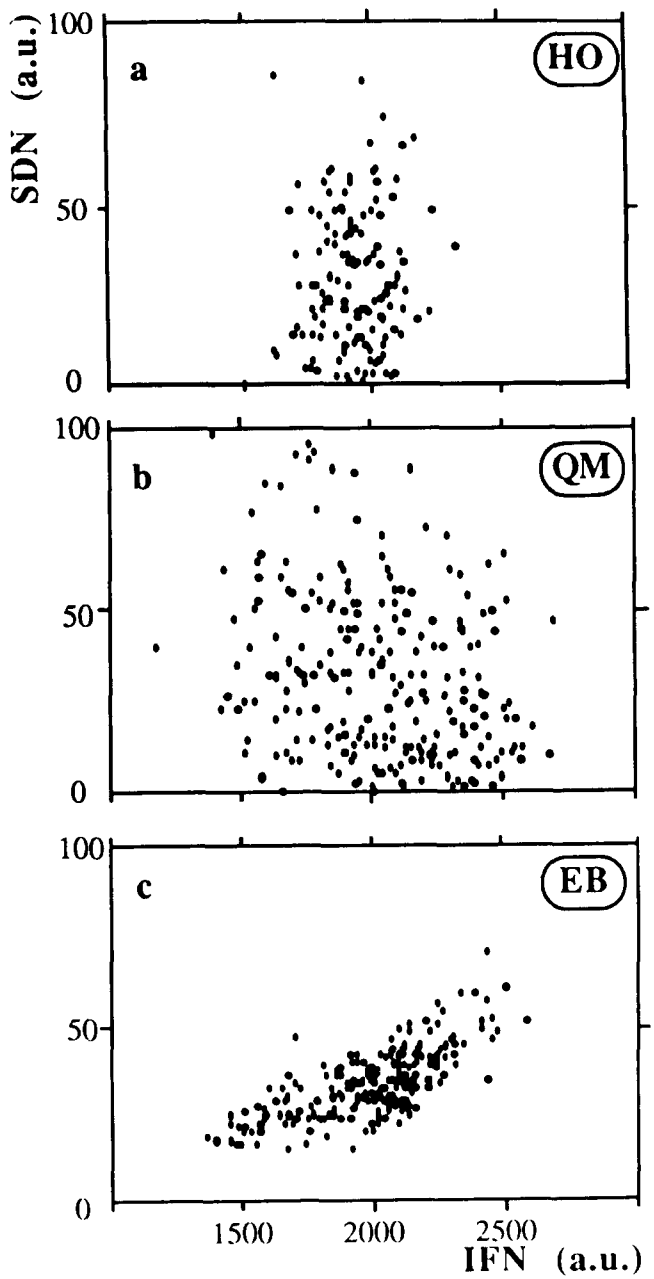

Figure 4. Scattergrams obtained for (a) HO, (b) QM, and (c) EB, showing examples of fluorescence intensities (IFN), and the variablity of the intranuclear distribution of the fluorescence intensities (SDN). a.u., arbitrary units.

Discussion

Ten fluorochromes among the most commonly used as DNA specific were compared to find those suitable to both quantitate the nuclear DNA content and analyze the chromatin pattern. To address this question, the DNA content and distribution of mouse hepatocytes were measured by image cytometry using these fluorochromes under identical fixation conditions known to preserve the chromatin structure.

Only HO and MA were found to provide satisfactory quantitation of nuclear DNA content as assumed by both a small CV and a 4c to 2c ratio equal to 2. PI, EB, 7-AMD, and OM provided higher CV values, although the 4c to 2c ratio was still equal to 2. QM, AO, CMA3, and DAPI provided non-reproducible and non-stoichiometric nuclear DNA content measurements under the fixation conditions used. It must be remembered that this fixation procedure is not conventional for fluorescence quantitation of DNA.

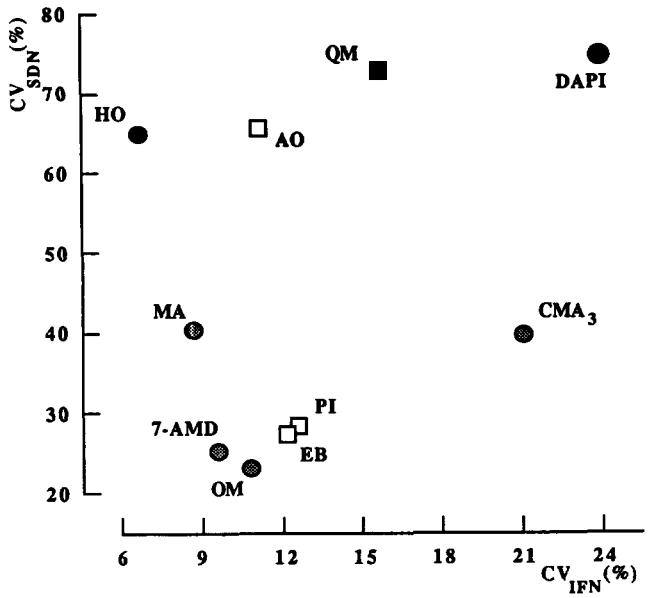

Figure 5. Scattergram of the 10 fluorochromes according to the variability of the nuclear distribution of the fluorescence intensities (CV_{SDN}) plotted against the variability of the DNA content (CV_{IFN}) among the 2c hepatocytes. Symbols as in Figure 2.

As far as DNA quantitation is concerned, ethanol is usually preferred to fixatives containing formaldehyde (like the Boehm–Sprenger used in this study). Conversely, only those formaldehyde fixatives have been proved to preserve the chromatin structure (Giroud and Montmasson, 1989). Studies that compare different fixation techniques do not exist yet for all the fluorochromes used in this study. Nevertheless, as had been suggested for MA (Crissman et al., 1978; Johannisson and Thorell, 1977), fixatives containing formaldehyde gave less satisfactory results for DNA measurement either with MA or DAPI (Coleman et al., 1981). Therefore, the differences reported here between the fluorochromes with regard to the DNA content measurement reproducibility and stoichiometry might result from the fixation procedure selected. It can be suggested that different fixatives alter in a different manner the DNA–protein configuration of the chromatin, thus resulting in a different binding mode of the fluorochromes to the DNA. In the case of QM, structural changes in chromatin organization can result in a different reciprocal distribution of dye molecules which, in turn, gives rise to different energy transfer efficiency and therefore to different fluorescence emission intensity (Andreoni et al., 1979). In this respect, significant differences in fluorescence intensities of chromosomes stained with QM have been found to be related to the DNA–protein interactions (Gottesfeld et al., 1974). Moreover, the obvious modifications of the QM fluorescence pattern and the decrease in quinacrine fluorescence throughout the G_1 phase of the 3T3 cell cycle have been also suggested to be due to the structural changes in chromatin and not to the quantity of bound fluorochrome (Moser et al., 1981).

Considering the fluorescence distributions obtained, a variety of chromatin patterns were observed according to the fluorochromes. This suggested that different fluorochromes stain DNA fractions that are different in many respects. The two parameters describing the fluorescence pattern of the 2c hepatocytes, i.e., the intranuclear and internuclear SD of the fluorescence, proved to vary according to both the BP specificity and the binding mode of the fluorochromes. Although the specificity and the mode of binding of all these fluorochromes have been largely debated (Barcellona and Gratton, 1990; Darzynkiewicz, 1979; Crissman et al., 1978; Ardnt-Jovin and Jovin, 1977; Krishan, 1975; Müller and Gautier, 1975; Le Pecq and Paoletti, 1967; Ward et al., 1965) the fluorescence pattern has never been taken into account to ascertain the actual mode of binding to DNA in situ. In this respect, the results reported here provide new information. For example, the similarity between the 7-AMD fluorescence pattern and that of the externally binding antibiotics (MA, CMA3, and OM), can be interpreted as resulting not only from their common GC specificity but also from a preferential external binding mode of the 7-AMD. Although the external and intercalating binding modes have been suggested for 7-AMD (Gill et al., 1975), the results reported here argue in favor of the external mode already reported to play an essential role in the stability of the DNA–7-AMD compound (Evenson et al., 1986; Balhorn et al., 1985). Another example is the similarity between the fluorescence pattern of QM-stained cells and that of AT-specific fluorochromes (HO and DAPI) with respect to both intra- and internuclear fluorescence distributions. The results reported here argue in favor of an increase in QM quantum yield when this fluorochrome is bound to AT, as already suggested by Pachmann and Rigler (1972).

The question arises of whether the measured DNA content is dependent on the chromatin pattern as the result of some measurement bias. At the cell population level no dependence was found, since the CV_{IFN} did not correlate with the CV_{SDN}. This means that internuclear variability of the fluorescence patterns does not result in variability of the DNA content. At the individual cell level, no correlation was found between the fluorescence distribution (SDN) and the measured DNA content (IFN) for HO, MA, QM, AO, CMA3, and DAPI. Among these dyes, HO and MA provided the most reproducible DNA content measurements and thus appeared very attractive for the analysis of internuclear distribution of AT- vs GC-rich DNA. With HO and/or MA, the chromatin pattern modification can therefore be analyzed together with DNA content in the course of such events as DNA replication, cell shifting from quiescence to cell cycle, or differential DNA transcriptional activity. QM, AO, CMA3, and DAPI are less adequate for simultaneous measurement of DNA content and analysis of chromatin pattern.

Interestingly, a significant correlation was found between the fluorescence distributions and the measured DNA content at the individual cell level for PI, EB, 7-AMD, and OM. The measured DNA content (IFN) increased with the fluorescence distribution heterogeneity (SDN) and this might result from a decreased fluorochrome accessibility to DNA. In the accessibility studies of Darzynkiewicz et al. (1984) and Evenson et al. (1986), the fluorochromes with intercalating binding mode already proved to have less accessibility than fluorochromes binding externally to DNA when the chromatin is condensed. In the case of 7-AMD, the limitation of accessibility with chromatin condensation is likely to result from the large size of this molecule as already reported by Darzynkie-

wicz et al. (1984) and Stokke and Steen (1987). In the case of OM, the limitation of accessibility with chromatin condensation is surprising, since it was not observed with the MA-related antibiotics. Although structurally related, OM and MA differ by minor variations in their chromophores and sugar components (Ward, 1965) and fluorescence emission (Crissman et al., 1978), which might correspond to different affinity features to DNA.

In conclusion, this study demonstrates that under conditions that preserve chromatin organization only two fluorochromes can be used to measure DNA content, i.e., HO for AT-rich DNA and MA for GC-rich DNA. With other fluorochromes, either the measured DNA content or the chromatin pattern is assessed in suboptimal conditions when fluorescent image cytometry is used.

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