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Accurate determination of DNA content in single cell nuclei stained with Hoechst 33258 fluorochrome at high salt concentration

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Summary. In an attempt to achieve accurate quantification of DNA levels in cell nuclei, we studied the influence of salt concentration on the fluorescence of cell nuclei complexed with Hoechst-33258 (Hoe) fluorochrome. The fluorescence of cell nuclei was compared with that of extracted DNA as well as that of nucleosome core. Conformational changes in these complexes were examined by measuring both fluorescence anisotropy and fluorescence lifetime in the nanosecond region. The results showed that the fluorescence of DNA-Hoe was quenched by the nucleosomal structure, there being an associated increase in anisotropy and a decrease in the fluorescence lifetime; however, the fluorescence was restored to the original level by the addition of a high concentration of NaCl, CsCl, or LiCl. The reduction in fluorescence may have been due to loss of fluorescence energy caused by collision of the fluorophore with histones in the nucleosome. The addition of 1 *M* NaCl to the medium used for staining with Hoe greatly stabilized the fluorescence of DNA in cell nuclei. The DNA content of individual cell nuclei was determined by comparing the fluorescence of these nuclei with that of a standard DNA solution. For lymphocytes and liver ploidy cells, reasonably accurate values were obtained by applying the present method.

Introduction

A rapid and very sensitive method is required to achieve accurate quantitative measurements of DNA levels in histochemical and cytochemical studies. Microfluorometry after the application of a reliable staining procedure using an appropriate fluorochrome has been found to be the most useful method for determining DNA levels in cells. Among the DNA fluorochromes previously examined, the bibenzimidazole dye, Hoechst 33258 (Hoe), has been widely used because of its high quantum efficiency and high specificity for DNA (Müller and Gautier 1975; Cesarone et al. 1979). This fluorochrome binds to adenine-thymine (A-T) regions of DNA strands, resulting in an increase in fluorescence intensity. When Hoe forms a complex with chromatin DNA, some nuclear proteins block its access to binding sites (Brodie et al. 1975; Latt and Wohlleb 1975), thus causing quenching of fluorescence intensity (Labarca and Paigen 1980; Downs and Wilfinger 1983). In microfluorometric assays of DNA using Hoe, this quenching is increased when the DNA structure is densely packed in the small volume of nuclei. Thus, the fluorescence level observed varies according to the condensation of chromatin, so that it is possible to use this dye in structural studies of DNA (Cowden and Curtis 1981). Therefore, before this fluorochrome can be used for microfluorometric measurements of DNA, the influence of higher DNA structure on Hoe fluorescence needs to be elucidated.

In the present study, we examined the relationship between the Hoe fluorescence and the conformation of the DNA-Hoe complex in cell nuclei. Conformational change can be detected by measuring fluorescence anisotropy (Araki et al. 1985). For the in situ anisotropic measurement of cell nuclei, we constructed a microfluorometer equipped with polarization optics. The fluorescence level and anisotropy value of cell nuclei were compared with those of extracted nucleosomes. From these results, we were able to determine the amount of DNA per single cell nucleus more accurately than is possible using existing procedures.

Materials and methods

Nucleic acids. Calf thymus DNA was prepared according to the method of Zamenhof et al. (1954) and was used as a standard. The concentration of DNA was determined from its absorbance at 260 nm, taking the molar absorption coefficient of DNA-phosphorus (DNA-p) to be as 6200 $M^{-1}\text{cm}^{-1}$. The nucleosome core (NC) and its purified DNA were prepared from isolated nuclei of rat ascites hepatoma cells (AH-130) as described by Tohno (1983). The DNA in the NC was assayed using the method of Burton (1956), with calf thymus DNA serving as a standard. Preparations of DNA and NCs were dissolved in 10 m*M* *Tris*-HCl buffer (*Tris* buffer), pH 7.2, before use. The base compositions (A-T %) of calf thymus DNA and rat ascites hepatoma DNA were 61% and 60%, respectively (Fasman 1976).

Fluorochrome. The bibenzimidazole dye, Hoe (Hoechst, Frankfurt, FRG), was used as a 200 μ*M* stock solution in water. This solution was stable for at least 2 months when kept cold in the dark. Dye solutions of the desired concentration were prepared by diluting the stock solution with *Tris* buffer before use. The concentration of dye was determined colorimetrically, taking the molar absorption coefficient at 338 nm as 4220 $M^{-1}\text{cm}^{-1}$ (Latt and Wohlleb 1975).

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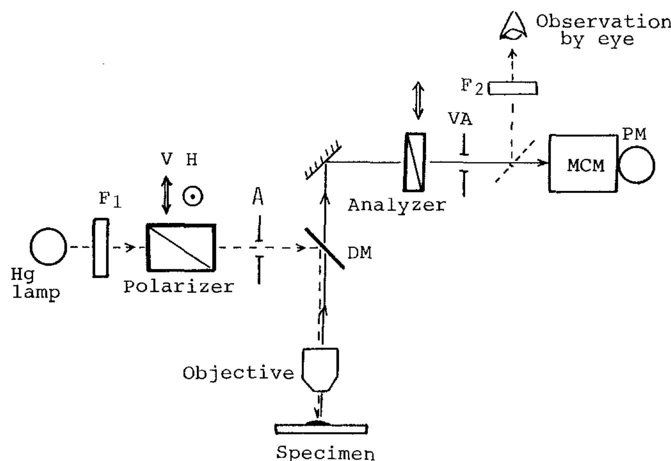


Fig. 1. Schematic diagram of the microfluorometer. F_1, band pass filter (350~370 nm); F_2, UV-cut filter; *A*, aperture to restrict the illumination area; *DM*, dichroic mirror; *VA*, variable-sized aperture to restrict the observation area; *MCM*, monochromator (set at 470 nm); *PM*, photomultiplier tube; *V* and *H*, polarized vertically and horizontally

Cell preparations. Lymphocytes and hepatocyte nuclei of adult male Wistar rats (body weight, 250 g) were used. Blood samples (5 ml) were withdrawn from the heart into a syringe containing two drops of anticoagulant solution (Anglot/ET; Nippon Shoji, Osaka, Japan). The lymphocytes were separated from the whole blood on a Ficoll-Hypaque (Flow Laboratories, North Ryde, Australia) according to the method of Ferrante and Thong (1980). Hepatocyte nuclei were isolated from liver tissue by using 7% citric acid. The resulting suspensions were smeared in a thin layers on glass slides, dried in air, fixed in absolute methanol for 5 min, and then dried again. The specimens were stained in *Tris* buffer containing 2 µ*M* Hoe and 1 *M* NaCl for 1 h at room temperature. A coverslip was placed on the specimen after the application of one drop of the staining medium, and it was then sealed on with rubber cement. Since the reactivity of Hoe dye with RNA is negligible (Brunk et al. 1979; Cowden and Curtis 1981), pretreatment with RNase was unnecessary. Fluctuations in the anisotropy measurements were reduced by using cover slips with a thickness of 0.15 mm (selected with a thickness gauge).

Fluorometry. We constructed a spectrofluorometer with a temperature-regulated (25° C) sample compartment (1 × 1 cm cuvette) and attachments for anisotropy measurements of extracted specimens. Using a Gran-Thompson polarizer, the excitation light was polarized vertically and horizontally against a vertically polarized analyzer for fluorescence, so that the fluorescence from the specimen was analyzed in the form of vertically and horizontally polarized components (*Fv* and *Fh*). From the values for these components, the total fluorescence intensity (*I*) and the anisotropy value (*A*) were calculated as follows:

$$I = Fv + 2Fh$$
$$A = (Fv - Fh)/I. \quad (1)$$

The anisotropy value is a function of the fluorescence lifetime (τ) and the rotational correlation time (Φ) of the fluorophore as follows:

$$A_0/A = 1 + (\tau/\Phi), \quad (2)$$

where A_0 is the anisotropy value measured in the absence of rotational motion for the diffusion process (Yguerabide 1971). The fluorescence lifetime and the rotational correlation time are defined as being the time constants of the decay of the total fluorescence intensity, *I*(*t*), and of the anisotropy, *A*(*t*), respectively. These were measured using a Horiba NAES-1100 nanosecond fluorometer.

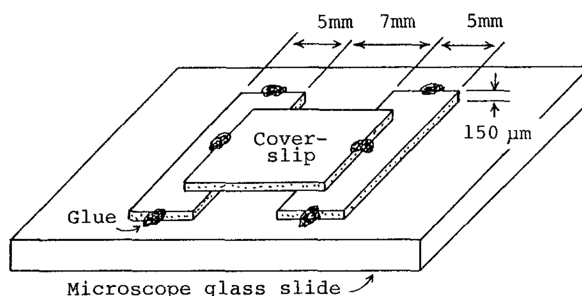


Fig. 2. Microcuvette used for determinations of DNA content per cell nucleus

The fluorescence of the DNA-Hoe complex was detected at an emission wavelength of 470 nm with excitation at 365 nm.

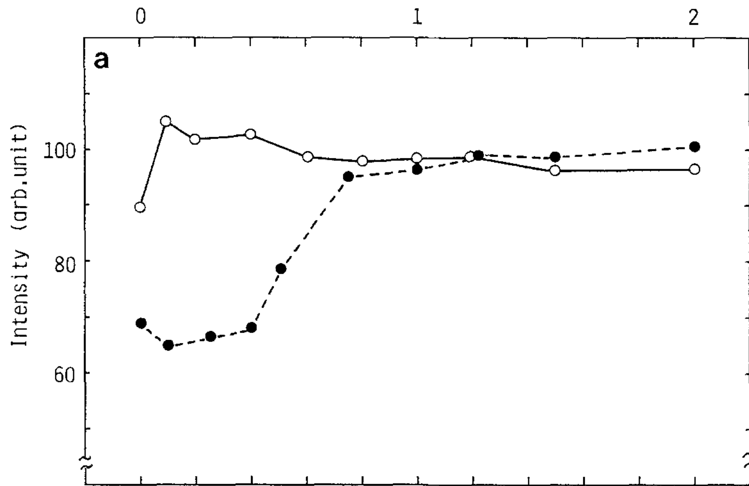
To measure the fluorescence anisotropy of the nuclear DNA-Hoe complex of single cells, we constructed an epi-illumination microfluorometer equipped with polarization optics (using a Nikon SPL fluorescence microscope as a base). The optical arrangement of this fluorometer is shown in Fig. 1. The excitation light was polarized vertically and horizontally against the vertically polarized analyzer. The total fluorescence intensity and the anisotropy value of the microfluorometric complex were obtained as in Eq. (1). A 365-nm mercury line was used as the excitation light of the complex, and the resulting fluorescence was detected at 470 nm. A ×40 objective (numerical aperture, NA = 0.85) was used for these measurements.

Quantification of nuclear DNA. The amount of DNA per cell nucleus was determined by calibrating the fluorescence intensity of single cells with that of a standard DNA-Hoe solution in a microcuvette. The microcuvette used in the present study was constructed from two parallel strips of coverslip (width, 5 mm; thickness, 150 µm) located 7 mm apart on a nonfluorescent glass slide, thus leaving 150-µm gap between the glass slide and the coverslip (Fig. 2). The microscope was focused on the middle layer of a DNA-Hoe solution in the cuvette. The image of the microcuvette was restricted by a variable aperture (*VA* in Fig. 1), and the size of this aperture was adjusted so that the fluorescence intensity of the standard DNA-Hoe solution was equal to that of the Hoe-stained nucleus being examined. The amount of DNA was determined by multiplying the concentration of the standard DNA by the observed cuvette volume [corresponding to the area of spot measured × cuvette thickness (150 µm)]. The cuvette used was fairly thick, and so a ×10 objective with a long focus depth (NA = 0.25) was used to avoid erroneous measurements due to defocusing of the cuvette. For DNA quantification, the polarization optics system was removed to obtain a brighter image.

Results

The influence of salt on fluorescence quenching

First, we compared the fluorescence of the DNA-Hoe complex with the NC-Hoe fluorescence. The concentrations of DNA-p and Hoe in the complexes were fixed at 40 and 1 µ*M*, respectively. The fluorescence intensity (*I*) of NC-Hoe was suppressed to 60% of that of DNA-Hoe in the presence of 0.15 *M* NaCl, at which NaCl concentration DNA is usually assayed (Fig. 3). At higher NaCl concentrations, the intensity and anisotropy (*A*) of the DNA-Hoe complex did not change. There was no difference between the fluorescence characteristics of Hoe with calf thymus DNA and Hoe with hepatoma DNA. The fluorescence of the NC-Hoe complex increased in intensity and decreased with respect to anisotropy, reaching the value of the DNA-



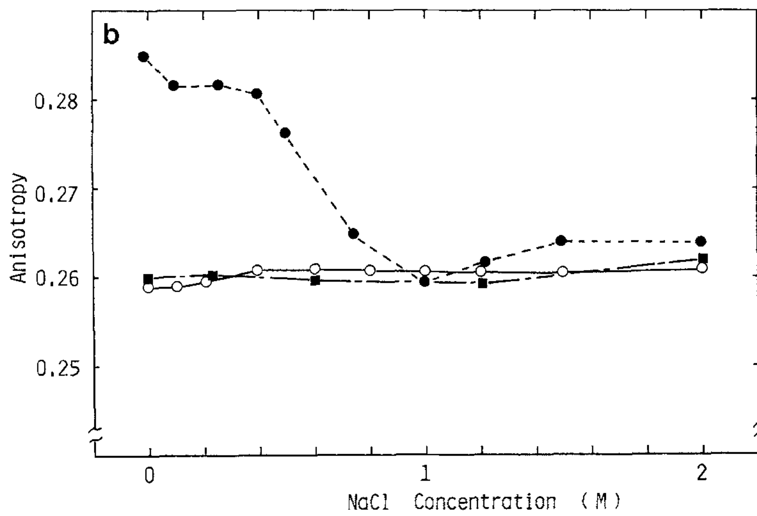


Fig. 3a, b. Fluorescence intensity (a) and anisotropy (b) of Hoe complexes with DNA and the NC as functions of NaCl concentration. o—o, calf thymus DNA; ■—■, rat hepatoma DNA; ●—●, hepatoma nucleosome core

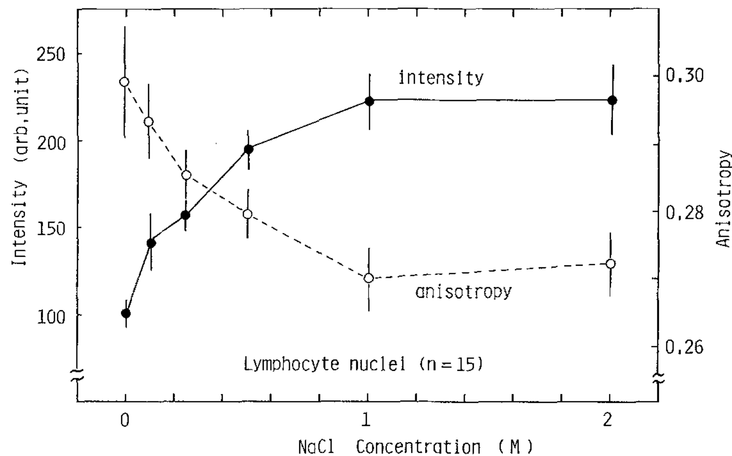


Fig. 4. Fluorescence intensity and anisotropy of lymphocyte nuclei stained with 2 μ*M* Hoe as functions of NaCl concentration

Hoe complex at NaCl concentrations of above 1 *M*. As with the fluorescence of the NC-Hoe complex, fluorescence intensity of the nucleus-Hoe complex increased with decreasing anisotropy when the NaCl concentration was increased up to 1 *M*, at which concentration the intensity level was more than double that observed in the absence of NaCl (Fig. 4). Thus, far less fluorescence emission was obtained under the usual staining conditions for microfluorometry, i.e., using low NaCl concentration. On the addition of 1 *M* NaCl to the Hoe medium, which would cause complete dissociation of histones H1 and H2 from nucleosomes, no quenching of the fluorescence was seen.

By measuring the τ and Φ values as in Eq. (2), we investigated why the nucleosome structure increased the A value and decreased the I value of the fluorescence. Figure 5 shows an example of the decay curve of total fluorescence

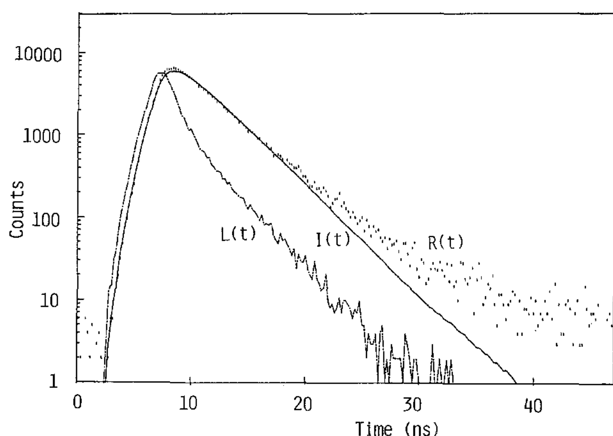


Fig. 5. Decay of fluorescence intensity of the NC-Hoe complex in the absence of NaCl. The actual decay curve, $I(t)$, was obtained from the raw data, $R(t)$, by deconvoluting the excitation lamp profile, $L(t)$

Table 1. Fluorescence lifetime and rotational correlation time of Hoe complexed with hepatoma NCs and with hepatoma DNA, and those of Hoe alone

Hoe complex	τ[ns]	Φ[ns]
Hoe alone	0.48	–[a]
DNA without NaCl	2.90	24.8
NC without NaCl	2.45	24.7
NC with 1.2 *M* NaCl	2.90	24.8

τ, fluorescence lifetime; Φ, rotational correlation time
[a] The correlation time of Hoe alone was too short to measure

Table 2. Influence of salt concentration on the fluorescence of the nucleus-Hoe complex

($n=15$)		Intensity	Anisotropy
Without salt		100±8	0.295±0.020
NaCl	0.2 *M*	126±7	0.291±0.010
	0.5 *M*	160±11	0.285±0.010
	1 *M*	180±13	0.270±0.012
LiCl	0.2 *M*	108±8	0.295±0.016
	0.5 *M*	117±12	0.288±0.022
	1 *M*	165±12	0.273±0.013
CsCl	0.2 *M*	130±10	0.289±0.024
	0.5 *M*	136±10	0.278±0.020
	1 *M*	178±14	0.269±0.012

Lymphocytes were stained with 2 μ*M* Hoe solution containing various salts

intensity [$I(t)$] of the NC-Hoe complex in the absence of NaCl. This curve was found to consist of two decay sections, i.e., a major rapid one and a minor slow one, when analyzed using a curve-fitting algorithm (Horiba NAES-1100); in contrast, the $A(t)$ profile of this complex consisted of more than two components. The $A(t)$ profile of the hepatoma DNA-Hoe complex was very similar to that of the NC-Hoe complex. The τ and Φ values were estimated by fitting single exponential functions to the major portions of $I(t)$ and $A(t)$, respectively, as shown in Table 1. The nucleosome structure did not change the rotational movement of the fluorophore, but did shorten its lifetime. We tried to confirm these values by investigating the complex in situ in cell nuclei, but we found no appreciable difference between the lifetimes in the presence and absence of NaCl, because of the poor temporal resolution obtainable using a transient microfluorometer (resolution time, 2.5 ns; Araki and Yamada 1985).

Table 3. Fluorescence intensity and anisotropy of lymphocyte nuclei stained with 2 μ*M* Hoe in the absence or presence of 1 *M* NaCl

($n=15$)	Intensity		Anisotropy	
	Without NaCl	With 1 *M* NaCl	Without NaCl	With 1 *M* NaCl
Unfixed	65±7	102±7	0.288±0.011	0.271±0.007
Fixed[a]	46±4	100±5	0.295±0.005	0.270±0.008
Fixed[b]	58±4	100±7	0.288±0.007	0.272±0.008

The fluorescence of fixed specimens was compared with that of unfixed ones.
[a] Stained immediately after fixation with methanol
[b] Stained 1 week after fixation with methanol

Quenching of the fluorescence of nuclear DNA-Hoe was also prevented by using other salts of monovalent cations, e.g., LiCl and CsCl. The effects of various salts on Hoe fluorescence are shown in Table 2, in which the fluorescence of lymphocyte nuclei in the presence of NaCl, LiCl, and CsCl are compared. At a concentration of 1 *M*, CsCl had the same effect as NaCl, whereas LiCl had less effect.

The effect of a high concentration of NaCl on the DNA assay was examined. As can be seen in Table 3, the fluorescence intensity varied according to the fixation used when cells were not treated with NaCl, and the differences with respect to anisotropy indicated that this variation was due to differences in DNA conformation, which was apparently altered by fixation and storage. In contrast, the fluorescence intensity was considerable stabilized by the addition of 1 *M* NaCl to the staining medium. After its addition, the fluorescence remained constant for at least 1 week, whereas the fluorescence from nuclei stained in the absence of NaCl was suppressed and then gradually increased (Fig. 6). This increase may have been due to the gradual dissociation of histones from nuclei.

Quantification of DNA in situ

We applied excess salt with Hoe for accurate quantification of DNA fluorescence in single cells. The quantum efficiency of the fluorescence of the DNA-Hoe complex varies according to the P/D ratio (molar ratio of DNA-p to fluorochrome). Therefore, it was necessary to know the P/D ratio in stained specimens in order to be able to quantify the amount of DNA from its fluorescence in the DNA-Hoe complex. We tried to determine the Hoe concentration in cell nuclei by colorimetry, but this was difficult, because the absorption of Hoe was too low to be distinguished from background absorption due to cell components. Therefore, we estimated the P/D ratio indirectly. As shown in Fig. 7,

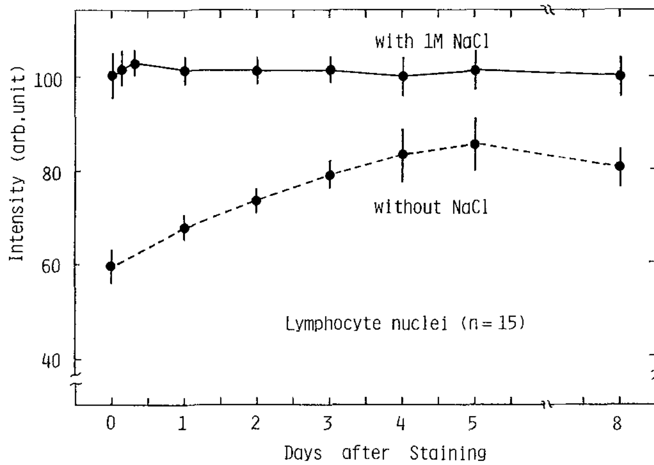


Fig. 6. Variations in the fluorescence intensity of lymphocyte nuclei with time after staining with 2 μ*M* Hoe. Results obtained using staining medium with and without 1 *M* NaCl are shown

in the presence of 1 *M* NaCl, the fluorescence intensity of the stained nuclei depended on the Hoe concentration until 2 μ*M*, at which dye concentration the fluorescence reached a plateau. As for staining in situ, the fluorescence intensity of the DNA-Hoe complex used as a standard reached a plateau at a P/D ratio of 10 in the presence of 1*M* NaCl (Fig. 7). From these results, the P/D ratio of in situ staining with 2 μ*M* Hoe was estimated to be 10. In the assays, the standard used was calf thymus DNA with a P/D ratio of 10 containing 1 *M* NaCl, and one drop of this standard was put in the microcuvette. The spot size on the cuvette was adjusted so that the fluorescence level of the standard DNA solution was the same as the level of fluorescence of cell nuclei.

Depending on the numerical aperture of the objective and the thickness of the cuvette, it seemed that the deeper and upper layers of the DNA-Hoe solution might not have been excited as much as the middle layer on which the microscope was focused, resulting in less fluorescence emission. To test this, we examined changes in the fluorescence level of a fluorescent thin plate placed in the off-focus plane of the microscope. As shown in Table 4, due to a small numerical aperture of the objective, the fluorescence intensity did not change appreciably, indicating that the present assay system is suitable for the determination of DNA levels in individual cell nuclei.

The reproducibility of this assay system was checked by calibrating the fluorescence level of the nuclei using standard DNA solutions of different concentrations (50, 100, and 150 ng/mm^3; equivalent to 143, 285, and 428 μ*M* DNA, respectively). As can be seen in Table 5, there was no appreciable difference in the DNA content of diploid (2c) hepatocyte nuclei as determined from the three standards. Using a standard solution with a DNA concentration of 100 ng/mm^3, the DNA content per lymphocyte nucleus was calculated to be 7.2 pg. The linearity between the amount of DNA and the fluorescence output was con-

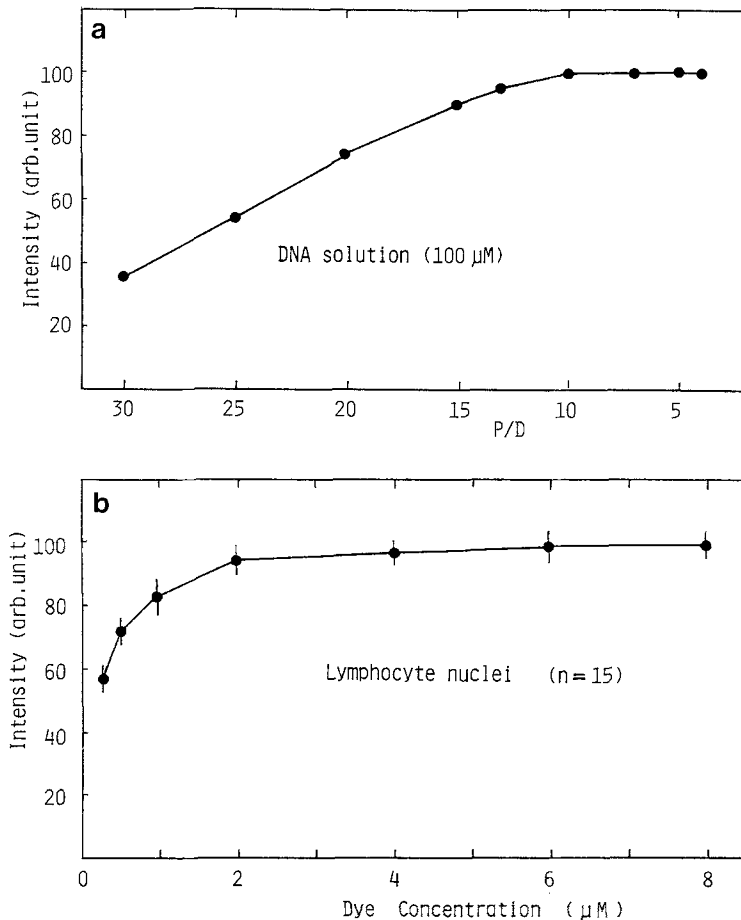


Fig. 7. a Fluorescence intensity of the DNA-Hoe complex (100 μ*M* DNA) as a function of the P/D ratio; **b** fluorescence intensity of lymphocyte nuclei stained with Hoe as a function of Hoe concentrations in the presence of 1 *M* NaCl

Table 4. Changes in fluorescence level as a function of the off-focus length of a ×10 objective

Distance between the objective and the surface of the fluorescent plate	Fluorescence intensity
f−75 μm	102
f−50 μm	100
f−25 μm	100
f	100
f+25 μm	98
f+50 μm	95
f+75 μm	90

f, focal length of the objective

Table 5. DNA content of single cell nuclei calculated by comparison with values in calf thymus DNA solutions

	DNA concentration in the cuvette [ng/mm³]	Aperture size on cuvette [μm²]	DNA content per nucleus [pg][a]
Hepatocyte (2c)	50	1020±40	7.7±0.3 (n=20)
	100	480±20	7.2±0.3 (n=20)
	150	325±12	7.3±0.3 (n=20)
Hepatocyte (4c)	100	965±35	14.5±0.5 (n=20)
Lymphocyte	100	480±25	7.2±0.4 (n=20)

Samples were stained with 2 μ*M* Hoe solution containing 1 *M* NaCl

[a] DNA concentration in cuvette × cuvette aperture size × cuvette thickness (150 μm)

Table 6. New procedure for efficient microfluorometry of DNA using Hoe fluorochrome

Smear free cells, nuclei, or tissue section on a glass slide and promptly air dry

↓

Fix in methanol (or Carnoy's fixative)

↓

Stain for 1 h with the NaCl-dye solution, i.e., 2 μ*M* Hoe in 10 m*M* *Tris*-HCl buffer, pH 7.2, containing 1 *M* NaCl (or CsCl, which may be better for preserving the microstructure of DNA)

↓

Place coverslip on the specimen without rinsing, and seal the coverslip with rubber cement

↓

Measure the fluorescence at 470 nm with excitation at 365 nm. The fluorescence is stable for at least 1 week in the dark

firmed by the finding that the amount of DNA measurable in tetraploid (4c) nuclei was exactly twice that in 2c nuclei. Since the base-pair compositions of rat and bovine DNA are very similar, the calculated values for DNA content should be within a few percent of the actual values. Moreover, our values were consistent with reported values, and especially those obtained by Downs and Wilfinger (1983) using Hoe as the marker fluorochrome. During illumination with excitation light, the fluorescence output from cell nuclei decreased by 5% per min, so that measurements for each nucleus were made within 10 s. The procedure applied for DNA assay in single cell nuclei is summarized in Table 6.

Discussion

The fluorescence intensity of Hoe increases markedly when it forms a complex with DNA. As with other fluorochromes such as ethidium bromide and acridine orange, the fluorescence intensity of the DNA-Hoe complex is mainly a function of the amount of DNA and is also affected by the secondary structure of the DNA. Thus, the fluorescence is greatly modified and decreased when Hoe is combined with DNA in cell nuclei. Therefore, it is essential, using microfluorometry, to determine whether variability in the fluorescence level reflects variation in the amount of DNA or in its structure. When assaying DNA in tissue homogenates, Downs and Wilfinger (1983) added ammonium hydroxide to the assay solution in order to remove nuclear proteins that affect the fluorescence. In the present study, we added excess salt to the staining medium to avoid the quenching of Hoe fluorescence, and we monitored the effect of salt on anisotropy.

When Hoe was reacted with nucleosomal DNA, the fluorescence intensity was 80% of that observed when the dye was complexed with an equivalent amount of free DNA; the reaction with nucleosomal DNA also resulted in 10% increase of anisotropy (Fig. 3). However, the presence of nonhistone protein did not influence the fluorescence (data not shown). At an NaCl concentration of 1 *M*, dissociation of histone H2 from NC is complete (Kleiman and Huang 1972) and NC DNA exhibits the same fluorescence as free DNA. Thus, the nucleosomal structure must be the main cause of the decrease in fluorescence intensity. The presence of NaCl during staining in situ also weakened the association of DNA with histone, resulting in a satisfactory recovery of fluorescence intensity (Fig. 4).

Why does depolarization occur on deformation of the nucleosome structure? Both the fluorescence lifetime and the rotational motion of the fluorophore are correlated with the stationary anisotropy value (A). We obtained the same Φ values (24.8 ns) for the three specimens by nanosecond fluorometry (Table 1). This value is almost the same as that obtained using ethidium bromide (28 ns; Wahl et al. 1970). The lifetime of NC-Hoe was shorter in the absence of NaCl than that in its presence. This finding indicates that histone does not restrict the motion of DNA in the nucleosome, but reduces the fluorescence lifetime. The rotational correlation time obtained in our study was smaller than that reported for the whole nucleosome particle (about 170 ns; Ashikawa et al. 1983), since the Φ value calculated from the fast-decay component only reflected the local motion of the fluorophore. Since a decrease in the fluorescence level was associated with a decrease in the lifetime, the fluorescence energy may have been lost as a result of collision of the fluorophore with histones inside the nucleosome. This collision probability decreases as histone dissociation increases. In the presence of 1 *M* NaCl, which results in the complete dissociation of histone H2, the loss due to collision is probably negligible, even though histones H3 and H4 are still present in the core particle. Thus, we found the level of fluorescence in the nucleosome-Hoe complex to be the same as that in free DNA-Hoe.

As in model experiments with extracted nucleosomes, we were able to observe the dissociation of histones in nuclei

in situ by measuring fluorescence anisotropy. In the presence of 1 *M* NaCl, the fluorescence intensity increased to twice that observed in the absence of NaCl. This increase was larger than expected, indicating that the fluorescence must be considerably suppressed by the packing of nucleosomes in nuclei (Redi et al. 1986). As DNA molecules are denatured when nuclei are fixed in formaldehyde solution (Dutt 1971), we used methanol for fixation; however even this relatively gentle procedure modified the nucleosomal conformation, resulting in variations in fluorescence intensity and anisotropy (Table 2). We often found that the fluorescence intensity of cell nuclei varied when several measurements were made in preparations subjected to conventional cell staining with DNA marker fluorochrome. This was not due to changes in the amount of DNA, but to changes in the nucleosome conformation before and after staining (Table 3, Fig. 6). In the presence of 1 *M* NaCl, the fluorescence level was very stable. Other monovalent cationic salts, e.g., LiCl and CsCl (Table 2), could also be used to stabilize such fluorescence, and urea was also found to be effective (data not shown).

We were able to determine the amount of DNA in individual cells by comparing their fluorescence intensity with that of a standard DNA solution. For this assay, the P/D ratio of the in situ stained cells must be equal to that of the DNA solution. As the P/D ratio of these cells was uncertain, we assumed it to be 10 when nuclei were stained with 1 *M* NaCl and 2 μ*M* Hoe (see Fig. 7). Müller and Gautier (1975) have reported that one Hoe molecule binds to three A-T base pairs of DNA. Since the A-T content of the DNA was 60%, it appears that one Hoe molecule also bound with three A-T pairs in our experiments, if the estimated P/D value (P/D = 10) was correct. We have previously shown that ethidium bromide is 60 times more concentrated in the nucleus than in medium, this being due to the affinity between ethidium bromide and DNA (Araki and Yamada 1985). Assuming the P/D ratio to be 10, the concentration of Hoe in the nucleus was calculated to be 3 m*M*, since the concentration of DNA in the nucleus estimated to be 30 m*M* using a microphotometric assay at 260 nm. Hoe would be more concentrated than ethidium bromide in the nucleus due to its stronger affinity to DNA. This needs to be confirmed in order to increase the reliability of our fluorometric assay of DNA in individual cell nuclei.

Using a microfluorometer equipped with an adjustable aperture, we measured the DNA content in individual nuclei and obtained a value of 7.2 ~ 7.7 pg per nucleus for rat 2c hepatocytes (Table 5). The use of a DNA solution with a concentration of as little as 50 ng/mm^3 required a large aperture, resulting in contamination with background emission and heterogeneous illumination, so that a higher value of 7.7 pg DNA per nucleus was obtained. The value for the DNA content of the hepatocytes (8.20 pg) reported by Higashi et al. (1966) was obtained by averaging the findings for 2c, 4c, and 8c cells of adult rat liver. Therefore, the DNA content of diploid cell nuclei would be 85% ~ 90% of this value. Our value of 7.2 pg DNA per nucleus for rat diploid cells obtained using a standard DNA solution containing 100 ng/mm^3 DNA is consistent with the value reported by Downs and Wilfinger (1983), who extracted DNA and determined its concentration by applying fluorometry using Hoe. In our study, we usually used a cuvette of 150 μm thick. However, this would be too thick when using a high-power objective with a short focal depth, so that a thinner cuvette would have to be used for measurements of small nuclei.

The measurements of DNA content in individual cells obtained using the present method were consistent with values determined by applying other methods requiring much larger numbers of cells. Our method would be especially useful when only a few cells are available. The degree of chromatin condensation in nuclei was assessed from the ratio of the fluorescence intensity in the absence of salt to that in the presence of a high-concentration salt. Thus, it was possible to estimate both the actual quantity of DNA and the degree of chromatin condensation in individual nuclei. The present method involving anisotropy measurement, which is both simple and rapid, should be helpful in studying the conformation of DNA in cell nuclei.

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