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Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants

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Flow cytometric estimation of nuclear DNA content was performed in six plant species employing three fluorochromes showing different DNA base preferences: propidium iodide (no base preference), 4′,6-diamidino-2-phenylindole (DAPI; AT preference), and mithramycin (GC preference). Nuclei isolated from human leukocytes were used as a primary reference standard. While nuclear DNA contents estimated using propidium iodide were in agreement with published data obtained using other techniques, the values obtained using fluorochromes showing base preference were significantly different. It was found that the differences were caused by the differences in overall AT/GC ratios, and by the species-specific differences in binding of these fluorochromes to DNA. It was concluded that nuclear DNA content estimations performed with fluorochromes showing base preference should be interpreted with caution even when AT/GC ratios of the reference and the sample are equal. The use of intercalating dyes (e.g. propidium iodide) is recommended for this purpose. On the other hand, comparison of the staining behaviour of intercalating dyes with that of dyes showing base preference may give additional information on chromatin structural differences and arrangement of molecule pairs in DNA.

Key words – DAPI, DNA base content, flow cytometry, mithramycin, nuclear DNA content, plants, propidium iodide.

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Introduction

Estimation of nuclear DNA content is one of the important applications of flow cytometry. The method is based on the isolation of single cells or nuclei in suspension and on the staining of nuclei with DNA fluorochromes. The fluorescence emitted from each nucleus is then quantified using a flow cytometer. Although the method was originally developed for the analysis of human and animal cells, it is now widely used also for plants (Galbraith 1989, Doležel 1991).

Fluorochromes currently used for flow cytometric estimation of DNA content can be broadly classified into two groups: stains that intercalate with double stranded nucleic acids and include ethidium bromide (EB) and propidium iodide (PI); and dyes and drugs that show a base preference and that include Hoechst 33258 (H33258), 4′,6-diamidino-2-phenylindole (DAPI), mithramycin (MI), chromomycin A3 (CH), and olivomycin (OL).

As flow cytometry provides only relative values, comparison with a reference standard having a known DNA content is necessary to determine picogram quantities of DNA. To make such a comparison valid, emitted fluorescence must be proportional to nuclear DNA content both in a reference standard and in a sample. While in the case of intercalators (EB, PI) binding to DNA is not affected by base composition (Le Pecq and Paoletti

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1967), DNA-specific dyes (H33258, DAPI) were shown to bind preferentially to adenine-thymine (A–T) rich regions of the DNA helix (Müller and Gautier 1975, Manzini et al. 1983), and DNA-specific drugs (MI, CH, OL) were shown to bind preferentially to guanosine-cytosine (G–C) rich regions of DNA (Ward et al. 1965).

It is generally agreed that fluorochromes showing base preference should not be used for estimation of nuclear DNA content in cases where base content of a reference standard and a sample differ (Coleman et al. 1981, Muirhead et al. 1985). Such a situation may frequently occur in plants where overall molar AT or GC content is not constant (Shapiro 1976). Nevertheless, while some authors use EB or PI to establish genome size in plants (Arumuganathan and Earle 1991, Michaelson et al. 1991a), others prefer to use fluorochromes showing base preference such as MI (Galbraith 1990, Kowles et al. 1990) or DAPI (Rayburn et al. 1989, Rayburn and Auger 1990). One of the probable reasons why base-preference fluorochromes are used is that the effect of base preference on flow cytometric estimation of nuclear DNA content has so far not been analysed in detail.

In this work, three commonly used DNA fluorochromes (PI, DAPI, MI) representing three different types of binding to DNA were compared for flow cytometric estimation of nuclear DNA content in six plant species covering a wide range of genome sizes.

Abbreviations – CH, chromomycin A3; DAPI, 4',6-diamidino-2-phenylindole; EB, ethidium bromide; H33258, Hoechst 33258; MI, mithramycin, OL, olivomycin; PI, propidium iodide.

Materials and methods

The plant material was derived from newly expanded leaves of young greenhouse-grown plantlets of radish (*Raphanus sativus* L.), tomato (*Lycopersicon esculentum* Mill.), maize (*Zea mays* L.), pea (*Pisum sativum* L.), broad bean (*Vicia faba* L.), and onion (*Allium cepa* L.). Human male leukocytes were used as a primary reference standard and chicken red blood cells were used for calibration of the flow cytometer.

To release plant nuclei, approximately 100 mg of leaf tissue were chopped with a sharp scalpel in a glass petri dish containing 1 ml LB01 lysis buffer (Doležel et al. 1989) of the following composition: 15 m*M* Tris, 2 m*M* Na_2EDTA, 80 m*M* KCl, 20 m*M* NaCl, 0.5 m*M* spermine, 15 m*M* mercaptoethanol, 0.1% Triton X-100, pH 7.5. Leukocyte nuclei were isolated by resuspending 6×10^6 fresh leukocytes in 1 ml ice-cold LB01 buffer. Because mithramycin (MI) requires magnesium ions for formation of complexes with DNA (Crissman et al. 1978), nuclei to be stained with MI were isolated using a modified LB01 buffer without Na_2EDTA and with 50 m*M* magnesium chloride and 30 m*M* sodium citrate.

The suspensions of released nuclei were passed through a 50-μm nylon mesh and stained in the dark for 60 min with 0.5 mg l^{-1} DAPI, or 50 mg l^{-1} MI, or 50 mg l^{-1} PI + 50 mg l^{-1} RNAase. The samples were filtered through a 15-μm nylon mesh just before measurement. The whole procedure was performed on ice.

Stained nuclei were analysed with a Leitz MPV-Compact flow cytometer. A 100-W high pressure mercury lamp was used for excitation. Filterblock A was used for analysis of DAPI fluorescence, filterblock H3 was used for analysis of PI fluorescence and filterblock N2 for analysis of MI fluorescence. Histograms of fluorescence intensity were registred over 512 channels. All histograms were evaluated using a Hewlett-Packard HP-86B microcomputer with FLOWSTAR software (Doležel 1989). At least 10000 nuclei were analysed in each sample.

Nuclear DNA content was estimated in relation to an assigned value of 7.0 pg DNA in fresh human leukocytes (Tiersch et al. 1989). The leukocytes were used as reference standard for the direct calculation of picogram quantity of DNA in *Z. mays* and *P. sativum*. Because genome sizes of other plant species were considerably different from that of leukocytes, *Z. mays* and *P. sativum* were used as internal reference for *L. esculentum* and *V. faba*, respectively. Similarly, *L. esculentum* and *V. faba* were used as internal reference for estimation of nuclear DNA content in *R. sativus* and *A. cepa*, respectively. This approach was undertaken to minimize the risk of error due to nonlinearity and the zero level error (Bagwell et al. 1989, Vindeløv et al. 1983).

The ratio of modal channel numbers of the G_0/G_1 peaks of the reference standard and sample nuclei (fluorescence ratio) was calculated. The ratio was corrected for zero offset error using the DNA ratio between single and double chicken red blood cell nuclei clumps (Givan et al. 1988). Each sample was analysed 5 times. The whole experiment was repeated 4 times. Statistical analysis was performed using Hewlett-Packard software.

Results

The effect of base preference of DAPI and MI on the analysis of nuclear DNA content was studied in 6 plant species. They were randomly chosen to cover a wide range in genome size. Optimal dye concentrations (giving the smallest coefficients of variation) had been previously determined in pilot studies. The coefficients of variation of G_0/G_1 peaks ranged from 2.9 to 4.8%, and were similar for all 3 fluorochromes used in this study.

Mean fluorescence ratios are listed in Tab. 1. The ratios obtained using PI differed from those obtained using either DAPI or MI. Genome sizes of selected plant species calculated using fluorescence ratios are listed in Tab. 2. The standard deviations of multiple independent measurements were very small (coefficients of variation did not exceed 3%) and therefore are not listed. In all 6 plant species, genome sizes estimated using DAPI differed significantly from those obtained

Tab. 1. Fluorescence ratios (fluorescence intensity of a sample/fluorescence intensity of a reference) of G_0/G_1 peaks obtained by flow cytometry using fluorochromes differing in DNA base preference.

Species	Fluorescence ratios (mean ± SD, n = 20)		
	Propidium iodide	DAPI	Mithramycin
R. sativus	0.343±0.005	0.604±0.007	0.488±0.014
L. esculentum	0.569±0.015	0.519±0.009	0.590±0.012
Z. mays	0.817±0.006	0.601±0.006	1.083±0.021
H. sapiens	1.295±0.026	1.432±0.017	1.329±0.025
P. sativum	2.968±0.086	3.068±0.048	2.946±0.037
V. faba	1.292±0.018	1.631±0.024	1.164±0.009
A. cepa			

using PI. The differences ranged from −26.4% for *Z. mays* to +44.3% for *A. cepa*. Similarly, the differences between genome sizes obtained using PI and MI, respectively, ranged from −8.2% for *A. cepa* to +96.4% for *R. sativus*. With the exception of *P. sativum* and *V. faba*, the values were again significantly different from those obtained using PI. As an example, the rank of PI, DAPI, and MI fluorescence intensities of *Z. mays* G_0/G_1 nuclei relative to leukocyte standard is depicted in Fig. 1.

To test the possibility that the differences were caused by the differences in DNA base composition, AT- and GC-contents were calculated using fluorescence ratios obtained with DAPI and MI, respectively. In these calculations, a linear base-dependent fluorescence of base preference dyes was assumed (Leeman and Ruch 1982). As shown in Tab. 3, large differences in overall AT/GC ratios were observed between individual plant species.

An unexpected finding of this study was that the nuclear DNA content calculated as a sum of AT- and GC-contents (AT + GC) differed from the DNA content estimated using PI. When the ratio (R) was calculated between the DNA content obtained as a sum of AT- and GC-contents and the DNA content obtained using PI, it was found to be different in each species (Tab. 3). This result indicated species-specific differences in binding of base preference fluorochromes to DNA.

The values of ratios R calculated for 6 plant species were based on the value of R = 1, arbitrarily chosen for human leukocytes (i.e. the 2C DNA content obtained using PI and that obtained as the sum of AT + GC were considered to be equal in human leukocytes). Thus, for instance, the value R = 0.974 obtained in *Z. mays* indicates that among the plant species studied the difference in binding of PI and base preference fluorochromes to DNA was smallest in this species. However, it does not necessarily mean that in *Z. mays* base preference dyes were bound less than PI. For instance, choosing the value of R = 1.5 for human leukocytes would result in the value of R = 1.461 for *Z. mays*. To describe a difference between a pair of species in binding of PI and in binding of base preference fluorochromes, a ratio K may be used. Its value is independent on the initial value chosen for the reference standard.

Theoretical considerations

Using flow cytometry, the genome size of any species can be estimated after simultaneous measurement of the fluorescence of stained nuclei of the species and of the reference standard with known DNA content. Let FR be the fluorescence ratio and GS_r and GS_s genome

Tab. 2. Genome sizes obtained by flow cytometry using propidium iodide (PI), DAPI, and mithramycin (MI). Human leukocytes (GS = 7.0 pg DNA) served as a primary reference standard. GS = genome size (2C nuclear DNA content in pg); $GS_{sample} = GS_{reference} \times$ fluorescence ratio. The means are based on 20 replicate measurements, the values significantly different ($P = 0.01$) from GS estimated using PI are marked by an asterisk *. References: (1) Bennett and Smith (1976); (2) Bennett et al. (1982); (3) Van't Hof (1965).

Species	PI	DAPI		MI		Published data	
	GS	GS	% PI	GS	% PI	GS	Reference
R. sativus	1.11	1.32*	118.9	2.18*	196.4	0.9	(2)
L. esculentum	1.96	2.54*	129.6	3.69*	188.3	2.0	(1)
Z. mays	5.72	4.21*	73.6	7.58*	132.5	4.7	(1)
P. sativum	9.07	10.02*	110.5	9.30	102.5	9.7	(1)
V. faba	26.90	30.75*	114.3	27.41	101.9	26.7	(1)
A. cepa	34.76	50.16*	144.3	31.90*	91.8	33.5	(3)

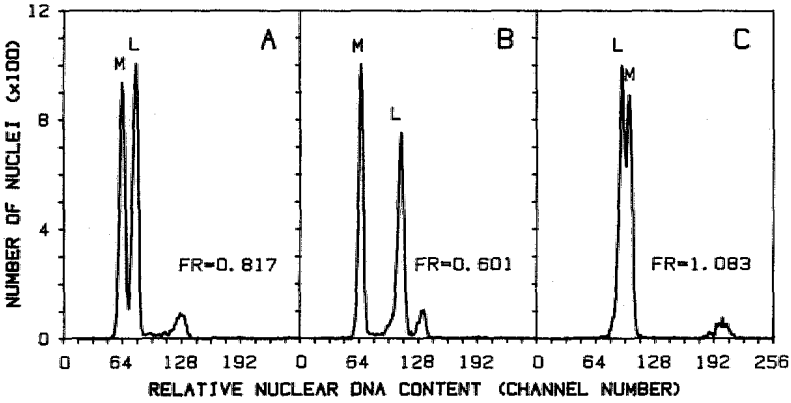

Fig. 1. Histograms of nuclear DNA content in maize (M) and human leukocytes (L) employing fluorescent dyes with different DNA base specificities: A, Propidium iodide; B, DAPI and C, mithramycin. Fluorescence ratio (FR) was calculated as a ratio of modal channel numbers of the G_0/G_1 peaks of leukocyte and maize nuclei. Only the first 256 channels of the histograms were plotted.

size of reference standard and of a sample, respectively. If the emitted fluorescence is proportional to DNA content the fluorescence ratio is equal to the ratio of the genome sizes

$$FR = \frac{GS_s}{GS_r} \quad (1)$$

and the genome size of a sample can be calculated as

$$GS_s = GS_r \times FR \quad (2)$$

In equations 1 and 2, the differences in overall AT/GC ratios are not considered. Therefore, these equations cannot be applied to those cases where a DNA fluorochrome exhibits base preference and where AT/GC ratios in the reference standard and in a sample are different. In such cases (assuming a linear relationship between the DNA base content and fluorescence intensity) the fluorescence ratio should be given by

$$FR = \frac{B_s}{B_r} \times \frac{GS_s}{GS_r} \quad (3)$$

where B_r is percentage base content (AT or GC) of the reference standard and B_s is percentage base content of a sample. From the data in Tab. 2 it is evident that equation 3 is not true because GS ≠ (AT + GC). In reality, the fluorescence ratio obtained using a base preference fluorochrome is given by

$$FR = \frac{B_s}{B_r} \times \frac{(AT + GC)_s}{(AT + GC)_r} \quad (4)$$

where $(AT + GC)_r$ is the sum of AT and GC content in the reference standard and $(AT + GC)_s$ is the sum of AT and GC content in a sample. Let $R_r = (AT + GC)_r/GS_r$ and $R_s = (AT + GC)_s/GS_s$. Substituting for $(AT + GC)_s/(AT + GC)_r$ in equation 4 yields

$$FR = \frac{B_s}{B_r} \times \frac{GS_s \times R_s}{GS_r \times R_r} \quad (5)$$

Let the coefficient K be $K = R_s/R_r$. Substituting for R_r/R_s in equation 5 yields

$$FR = \frac{B_s}{B_r} \times \frac{GS_s}{GS_r} \times K \quad (6)$$

Tab. 3. Comparison of genome size with the sum of AT and GC contents estimated using flow cytometry. Human leukocytes (AT = 59.5%, Shapiro 1976) were used as a primary reference standard. GS = genome size in pg DNA; FR fluorescence ratio; $AT_{sample} = AT_{reference} \times FR$; $GC_{sample} = GC_{reference} \times FR$; AT (%) = AT / (AT+GC) × 100; GC (%) = GC / (AT+GC) × 100; R = (AT + GC) / GS; $K = R_{sample} / R_{reference}$.

Species	GS	AT (pg)	AT (%)	GC (pg)	GC (%)	AT+GC (pg)	R	K
R. sativus	1.11	0.78	46.99	0.88	53.01	1.66	1.495	0.97
L. esculentum	1.96	1.51	50.17	1.50	49.83	3.01	1.536	1.57
Z. mays	5.72	2.50	44.88	3.07	55.12	5.57	0.974	0.97
H. sapiens	7.00	4.17	59.50	2.83	40.50	7.00	1.000	1.07
P. sativum	9.07	5.97	61.42	3.76	38.58	9.72	1.072	1.02
V. faba	26.90	18.32	62.31	11.08	37.69	29.40	1.093	1.12
A. cepa	34.76	29.88	69.85	12.90	30.15	42.78	1.231	

and thus the genome size of a sample can be calculated as follows

$$GS_s = GS_r \times FR \times \frac{B_r}{B_s} \times \frac{1}{K} \quad (7)$$

This conclusion has serious implications. It means that the genome size of an unknown sample (GS_s) cannot be correctly estimated using a base preference fluorochrome unless the value of the coefficient K and the overall AT/GC ratios in the reference standard and the sample are known.

Discussion

The precision and rapidity of flow cytometric estimation of nuclear DNA content makes the method very attractive for estimation of genome size both in animal and plant species. A range of DNA fluorochromes can be used to stain nuclear DNA (Muirhead et al. 1985). While some of them intercalate quantitatively between base pairs of double stranded nucleic acids, others bind selectively to AT- or GC-rich regions. Insensitivity of DNA intercalators (such as EB or PI) to base composition makes them very suitable for comparative studies of DNA content. This was confirmed by the present study as 2C nuclear DNA contents estimated using PI in all 6 plant species were very close to values obtained using Feulgen microspectrophotometry (Tab. 2).

A good correlation between the DNA values of 10 plant species determined by flow cytometry using PI and by Feulgen cytophotometry was also observed by Michaelson et al. (1991b). Nevertheless, their values for *P. sativum* and *V. faba* differ by about 15% both from the data obtained by microspectrophotometry and from our data. Although such discrepancies may be due to the use of different cultivars of the species, they may be also due to use of improperly calibrated reference standards. The ultimate solution of this problem would be to select a range of standards covering a whole range of genome sizes in plants and to calibrate them against a fundamental standard using a reliable chemical assay.

Our results clearly demonstrated that the use of base preference fluorochromes may lead to serious errors in the estimation of genome size in plants by flow cytometry. In most cases, the differences between DNA contents obtained using PI and those obtained using DAPI or MI were statistically highly significant. We have suggested that these differences were caused by the differences in DNA base composition and by the differences in binding of PI and base preference fluorochromes to DNA.

Throughout this study, a linear relationship between fluorescence and base content was assumed for base preference fluorochromes. However, there is no general agreement on this relationship. The results of Leeman and Ruch (1982) obtained with DAPI and chromomycin A3 suggested a linear or close to linear relationship. Assuming a linear dependence, Kubbies and Friedl (1985) estimated AT-content in human and bovine cells. Their results were in accordance with published data. Similarly, our estimates of AT- and GC-contents are also in accordance with data obtained using other techniques (Shapiro 1976, Stack and Comings 1979, Leeman and Ruch 1982).

Recently, Brown et al. (1991) suggested a curvilinear relationship between base content and fluorescence intensity of base preference dyes. However, DNA base contents calculated according to their model using our fluorescence ratios differed to various extents, from both our results and published data. For instance the GC-content of *Allium cepa* calculated according to Brown et al. (1991) assuming 4 GC pairs as a binding site for MI, was equal to 39.64%. This value differs from our result of 30.15%, and also from published values (31.6 and 33.2%) obtained using two different methods (Stack and Comings 1979). Clearly, further research is needed to clarify the relationship.

Theoretically, the use of fluorochromes showing base preference should not disturb estimations of nuclear DNA content in cases when overall AT/GC ratios in the reference standard and in a sample do not differ (Schnedl et al. 1977, Brown et al. 1991). In contrast, the present study showed that the use of base preference fluorochromes may lead to erroneous estimations even in these cases. The fact that the ratio R of the sum of AT + GC content to the 2C DNA content estimated using an intercalator was different for each species indicated species-specific differences in binding of base preference fluorochromes to DNA. Therefore nuclear DNA content cannot be estimated correctly using base preference fluorochromes even in cases where overall AT/GC ratios in the standard and the sample are equal.

The nature of these differences is not clear. Darzynkiewicz et al. (1984, 1988) have shown that a large proportion of nuclear DNA in native chromatin is unstainable, and that the amount of unstainable DNA varies depending upon chromatin structure. To avoid a possible effect of chromatin structure, we have isolated cell nuclei from the same type of tissue (newly expanded leaf of young plantlets) in all 6 plant species. However, as gross organization of chromatin in plants was shown to be species-specific (Nagl 1982), we cannot exclude a possibility that the differences in binding of base preference fluorochromes observed in this study were caused by the species-specific differences in chromatin structure.

Another factor to be considered is the effect of the arrangement of molecule pairs in DNA. For instance the binding of DAPI in the minor groove of DNA requires 3–4 consecutive AT base pairs (Portugal and Waring 1988, Wilson et al. 1989). Thus the differences in binding of base preference fluorochromes to DNA observed in this study may reflect species-specific differences in base pair arrangement (for instance in the case of DAPI, the differences in the number of AT-rich

binding sites). This conclusion is supported by the results of Kubbies and Friedl (1985) who observed species-specific differences in quenching of H33258 fluorescence by BrdU-substituted chromatin.

To conclude, the present study clearly shows that flow cytometric estimations of nuclear DNA content in plants performed with fluorochromes showing base preference should be interpreted with caution. Moreover, due to species-specific differences in binding of these fluorochromes to DNA found in this study, even the use of a reference standard having the same AT/GC ratio as the sample cannot guarantee a reliable result. On the other hand, comparison of data obtained using DNA intercalators with those obtained using base preference fluorochromes may give additional information on chromatin structure and/or arrangement of molecule pairs in DNA.

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