Genome size estimation: a new methodology.

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ABSTRACT

Recently, within the cytogenetic analysis, the evolutionary relations implied in the content of nuclear DNA in plants and animals have received a great attention. The first detailed measurements of the nuclear DNA content were made in the early 40's, several years before Watson and Crick proposed the molecular structure of the DNA. In the following years Hewson Swift developed the concept of "C-value" in reference to the haploid phase of DNA in plants. Later Mirsky and Ris¹ carried out the first systematic study of genomic size in animals, including representatives of the five super classes of vertebrates as well as of some invertebrates. From these preliminary results it became evident that the DNA content varies enormously between the species and that this variation does not bear relation to the intuitive notion from the complexity of the organism. Later, this observation was reaffirmed in the following years as the studies increased on genomic size, thus denominating to this characteristic of the organisms like the "Paradox of the C-value". Few years later along with the no-codification discovery of DNA the paradox was solved, nevertheless, numerous questions remain until nowadays unfinished, taking to denominate this type of studies like the "C-value enigma". In this study, we reported a new method for genome size estimation by quantification of fluorescence fading. We measured the fluorescence intensity each 1600 milliseconds in DAPI-stained nuclei. The estimation of the area under the graph (integral fading) during fading period was related with the genome size.

Keyword list: image processing, genomic size, fluorescence intensity.

1. INTRODUCTION

The genomic size in eukaryotes varies more than 200.000 times including the protists. In animals, the rank is approximately of 2.500 times². At the moment, the genomic sizes of approximately 3000 animals and near 4000 plants, as well as a variety of fungi, protists and bacteria have been described^{2, 3}. The knowledge of the genomic size of a species is not only relevant in general terms of biological questions, but also it can be useful in the classification of organisms jointly with the cariological information of a species. Probably nowadays in the climate of molecular biology, the most important aspect of the genomic size is that this one is an important factor for the development of future genomic projects of sequences, as well as for studies of genomic structure and evolution⁴.

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The relevance of the data obtained through the study of genomic size has caused an intensification in the development of methodologies able to consider in a more precise way the amount of nuclear DNA, with which, several methods have been used to quantify nuclear DNA⁵.

Some of the first methodologies of estimation of genomic size were based on techniques of genomic DNA extraction. Nevertheless, this technique was reported as vague because it was necessary to know the agreement cellular from which the process of extraction of the DNA had been made⁵. Later, these techniques of extraction were replaced by densitometric methodologies, which are based on the quantification of the coloration of samples submitted to Feulgen reaction. Briefly, the densitometric techniques have the premise that the intensity of coloration is directly proportional with the amount of present DNA. The amount of stain is determined by means of the amount of light that is absorbed (density), nevertheless, the quantification of density is not possible to be made directly, with which it must be considered indirectly through the transmittance. The transmittance of a sample must be calculated by means of the difference between the incident light that passes through an object (for example a nucleus) and the transmitted total light⁵. Due that the densitometric methods at the moment are very used to consider genomic size⁶, because they have integrated some procedures of image analysis, the main disadvantage is that when light goes through a sample will be affected by the phenomena of the light like diffraction, reflection and refraction, with which the precise measurement of the total transmitted light could affect the quantification of the absorbance or density.

Like an alternative to the densitometric stains, it is possible to quantify the fluorescence intensity by means of the use of specific stain for DNA. In this sense, the fluorometry has been developed so much for static measurements or direct quantification in a single plane or dynamic measurements as in the case of the flow cytometry. In the case of static measurements, special microscopes have been developed to directly measure the fluorescence from cellular samples, with which it has been possible to determine ploidy levels of spermatozoa and polyploidy levels in polyploid organisms^{7, 8}. In reference to the flow cytometry, probably it is the technique with greater acceptance and diffusion as far as measurement of genomic size⁹. From its development at the end of 70s to detect DNA anomalous contents in cancerogenic cells, the flow cytometry has become essential in genomic size research of animals and plants^{10, 11}. Briefly, this method consists of dealing with a sample cells suspended with fluorochrome DNA-specific. The measurement of the fluorescence is made by means of the incidence of a beam of laser light on a flow of cells dyed with fluorochrome¹². This technique has been described as fast and with good precision¹³. Nevertheless, the disadvantage of this technique is that requires a treated sample to obtain nuclei in suspension and a large number of nuclei for analysis.

In observation by conventional fluorescence microscopy, several DNA-specific fluorochromes (e.g. DAPI, Propidium Iodide, Ethidium Bromide, Hoechst 33258, etc) the fluorescence is rapidly lost when it is exposed to excitation light¹⁴. The fading is a phenomenon, which occurs specifically for each type of fluorochrome at specific time. But, is this fading time independent or dependent to genome size?, to our knowledge this has not been studied in detail. The aim of this work is to determine a new method for genome size estimation by quantification of fluorescence fading using image analysis.

2. Material and methods

2.1 Biological material

To determine the relationships between the DNA-fluorescence fading time and the genome size, we used biological material of known C-value as standard¹⁵. Diploid and haploid cells were used for fluorescence fading measurement: spermatozoa and red blood cells of Mozambique tilapia (*Oreochromis mossambicus*), spermatozoa of red abalone (*Haliotis rufescens*) and red blood cells of rainbow trout (*Oncorhynchus mykiss*). All biological material was obtained from stocks of the Aquaculture Department, CICESE, Baja California, Mexico.

2.2 Sample collection and cell preparation

Fish blood of Mozambique tilapia and rainbow trout were collected by brachial arc puncture after the fish anaesthetized with hydroxyl-phenol (0.5 mg/ml and 0.2 mg/ml respectively). Monolayer of red blood cells was smear on clean slides according to Hardie et al⁵. After air drying, the preparation was fixed in absolute methanol for 20 minutes and stored in

methanol at 4°C. After fixation the slides were passed through ascending grades of alcohol (70%, 90% and 100%), two changes of each for 5 min.

For obtaining of spermatozoa from Mozambique tilapia, fishes in reproductive stage were induced to sperm release by abdominal rub down. The sperm was smear and fixed on clean slides with fresh Carnoy (Methanol: acetic acid, 3:1) at 4°C and after air drying. For obtaining samples of spermatozoa in red abalone, adult organisms were induced to spawn according to Morse et al.¹⁶. The smear slide was obtained by similar procedure that Mozambique tilapia sperm.

2.3 DNA-fluorescence stain

The fixed cells on slides were washed three times with Phosphate Buffer Saline (PBS) for 15 min and incubate with DAPI solution in dark for 25 min at room temperature. The DAPI solution stain was prepared with 4,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical; St. Louis, MO) in PBS at 0.5 µg/ml concentration. This concentration allowed obtaining accurate fluorescents images without cell overstaining. The fluorescence was measure using an epifluorescent microscopy Leica model DMRXA2, equipped with a RGB color digital camera of 36-bits Leica model DC300 (3.3 Mega Pixels). The conditions were; Excitation filter UV 340-380 nm, Dichromatic mirror DM 400 nm, Suppression filter LP 425, Absorption Band AB 435-485 nm and objective lent PL Fluotar 63X/0.7.

2.4 Image capture and fluorescence fading analysis

The fluorescent images were digitally captured with a macro programmed under QWIN software (Copyright © by Imaging Systems Ltd, Cambridge, U. K., 1997), and connected to a motorized epifluorescent microscopy Leica DMRXA2, plus digital camera Leica DC300. This program sequentially captures digital images during a fluorescent fading period upon the slide. In addition, the program is able to perform an automatic capture of several microscope fields on the examined slide. Thus, it is not necessary pre-locate the cells to measure the fluorescent fading; moreover the best focus is initially positioned. The capture condition were; CCD exposure time of 509 millisecond, gamma equal to 1 (linear response) and color deep of 8 bit/channel.

Image analysis was performed by an algorithm specifically built in MATLAB software (Copyright © 1984-2000, The MathWorks, Inc.). The figure 1 shows the flow diagram representing the steps of the algorithm to obtain the fluorescent fading values. In the step 1, $f_1, f_2, \dots, f_k$ is the stack of k images sequentially captured of size $N \times M$ pixels. The step 2 shows the fluorescence measurement in a single nucleus. The fluorescence intensity was estimated by means of a pixel mean intensity (PMI), where $Q(x, y)_i$ is the image matrix in the i 'th nucleus, and $Q \in f$ such as a subimage of $f_1, f_2, \dots, f_k$. The image mean was calculated by $\frac{1}{n} \sum_{i=1}^n Q(x, y)_i$, where n represents the number of $N \times M$ pixels. The fluorescence intensity was indexed at 256 level of brightness. In the step 3, we calculate the fluorescent fading during the bleaching time by estimation of the integral or $IF = \sum_{k=1}^n (PMI)_k * \Delta t$, where the subscript k represents the number of images in a fading time and Δt the elapsed time between consecutive images. In the step 4, we graphically show the integral fading like the area under the curve. Thus, IF represents the integral fading by the sum of all PMI from each nucleus through the fading process.

3. Results

3.1 The nature of the decrease in DAPI-fluorescence

To determine the relationship between DAPI-fluorescence fading and genome size, we compared by the time course of bleaching of DAPI-stained nuclei in spermatozoa and red blood cells of Mozambique tilapia, spermatozoa of red abalone and red blood cells of rainbow trout. To visualize the fluorescence fading period, we captured twelve images of DAPI-

stained nucleus of Mozambique tilapia among 1.6 sec to 192 sec at different intervals. The sequence of images in DAPI-stained nucleus showed that the fluorescence fading period decreases rapidly when these nuclei were U.V light exposures until total bleaching.

Fig. 1 Flow diagram representing the steps of the algorithm to obtain the fluorescent fading values.

Fig. 2. Sequence of images in DAPI-stained nucleus of Mozambique tilapia, *Oreochromis mossambicus*. The sequence shows the fluorescence fading period in a nucleus of red blood cell, U.V light exposure between 1.6 sec and 192 sec. The number of images captured between the first and last image was of 120 images (in the chart only shows twelve images). The color bar indicates 256 level of brightness or fluorescence intensity from 0 to 255.

Fig. 3 Profile of DAPI-fluorescence fading for spermatozoa in tilapia. The fluorescence fading were measured each 1.6 seconds until total fading.

The fluorescence intensity or brightness level was visualized by comparison with a color bar. This bar shows the pixel intensity in 256 level of brightness (Fig. 2).

To compare the fading period in different species, we obtained the profile of DAPI-fluorescence fading in spermatozoa (EZT) and red blood cells (RCT) of Mozambique tilapia, spermatozoa of red abalone (EZR) and red blood cells of rainbow trout (RCTr). The profiles showed a decrement of the fluorescence intensity (FI) as a function of time. The maximum value of intensity is found in the first exposures time of the U.V light. The profiles variability in different fields showed that the major data dispersion were mainly produced within the 20 seconds of exposures to U.V. light, and after this time the fading profile became more stable independently of field number. The initial fluorescence intensity in the different nuclei types analyzed to explain the relation between fluorescence intensity and genome size. However, the fluorescence fading decrease differentially according to nuclear type studied. An example of these profiles is shown in the figure 3.

The DAPI-stain fluoresces mainly in the blue color interval, however in digital images, color is formed by contribution of the three color channels; red (R), green (G) and blue (B). The information of the RGB channels in terms of fluorescence intensity was verified by extraction of R, G and B channels. To visualize the RGB channels intensity in DAPI-stained nucleus of spermatozoa of red abalone, we extracted each channel from the first image of the fading period. These images (R, G and B) showed that the B channel is the main contributor with the highest brightness information or fluorescence intensity and in a lesser degree both R and G channels.

To determine whether this contribution of RGB channels was similar in the cells types studied and independent to genome size, we tested the decomposition channels in fading profiles. For each channel (R, G and B), fluorescence intensity was plotted against time and the RGB channel (true color) was obtained by mean the three channels. An example for spermatozoa of Mozambique tilapia (Ezt) is shown in the figure 4. These analyses showed that during the fading period the B channel is the main contributor of brightness and both R and G channels contributed in a lesser degree. An important observation is that G channel was placed in the mean position among the three channels, in this case, it would be possible to work with only G channel because it could be representative of mean channel. However, only to work with G channel increases the compute time, because is necessary to generate an additional estimation to extract the G channel from RGB channel. In this study, we worked with RGB channel (true color) which represented the mean of the red, green and blue channels and therefore, it is not require an additional processing.

Fig. 4 Fading profiles of DAPI-fluorescence in RGB channels for spermatozoa of Mozambique tilapia (Ezt)

3.2 The relationship between DAPI-fluorescence fading and genome size

The fading profiles were used for estimation the area under the graph, this area represents the integral fading (IF). To ascertain the hypothesis in this study, that IF is function of the genome size. We plotted the integral fading of spermatozoa and red blood cells nuclei of Mozambique tilapia. Both of them have different value of integral fading,

however the spermatozoa IF value was 1416.58 and this is equivalent to half value the IF in the red blood cells, equal to 2825.90. This relationship represented both haploid and diploid IF values for this species (Fig. 5).

Fig. 5 Fluorescence fading profiles of spermatozoa (EZT) and red blood cells of Mozambique tilapia (RCT). The area under graph represents the integral fading (IF). The IF value of spermatozoa (IF = 1416.58) was equivalent IF half value the red blood cells (IF = 2825.90). This relationship represents both haploid and diploid IF values for this species.

Additionally, we calculated the number of nuclei (sample size) required to estimate the IF value representative for each species studied. Several fields were measured to count a number major of 150 nuclei per species. The IF value per nucleus was randomly ordered for calculate the variance and mean IF. These descriptors were estimated by increasing the number of counter nuclei from 2 to n nuclei. Numerically we found that the variance and mean IF values beginning to be steady after 100 measured nuclei. Therefore, when the variance and mean IF were steady, in this point was considered the representative IF value for each nuclear type. The IF values found for spermatozoa (EZT) and red blood cells (RCT) of Mozambique tilapia, spermatozoa of Pacific red abalone (EZR) and red blood cells of rainbow trout (RCTr) are shows in the Table 1.

To calculate the genome size we determined two ways. In the simplest approach, a proportional directly relationship of IF vs known C-value:

$$DNASu = DNASs \times \left(\frac{IFu}{IFs} \right) \quad (1)$$

where $DNASu$ = unknown genome size, $DNASs$ = known genome size (standard), IFu = unknown mean IF, and IFs = standard mean IF. Alternatively, we used the regression equation of the standard curve for calculated genome size. From linear model equation $\hat{y}=3337.2\hat{x}+596.8$ was obtained the relation following:

$$DNASu = \left[\frac{(IFu - \hat{\beta})}{\hat{\alpha}} \right] \quad (2)$$

where $DNASu$ = unknown genome size, IFu = unknown mean IF, with $(\hat{\beta})$ and $(\hat{\alpha})$ equal to y -intercept and slope in the linear regression (Fig. 6).

Table 1. Integral fading (IF) values and DNA content calculated for each species and C-value obtained by references.

Standard	Number of nuclei	Integral fading (IF)	DNA content (pg)†	C-value (pg)	Reference
EZT	160	1416.58	0.91	0.81 1.00	Cui et al. 1991 Majumdar and McAndrew, 1986
RCT	245	2825.91	1.58*	-	-
EZR	180	3080.28	1.71	1.8	Hinegardner, 1974
RCTr	360	9642.80	4.83*	2.40 2.75 2.85	Hardie and Hebert, 2003 Peterson et al. 1994 Vindeløv et al. 1983

* Diploid value of DNA content (2C).

† DNA content calculated by linear model.

According the linear model we determined the genome size for spermatozoa and red blood cells of Mozambique tilapia (*Oreochromis mossambicus*) in 0.91 pg and 1.58 pg respectively. The genome size in spermatozoa of red abalone (*Haliotis rufescens*) and red blood cells of rainbow trout (*Oncorhynchus mykiss*) were determined in 1.71 and 4.83 pg respectively. The genome size calculated for rainbow trout represent the diploid value for this species, therefore the haploid value corresponding is 2.415 pg (Table 1). To determine the confidence intervals at 95% we calculate by Fisher's analysis the mathematical expectation $E(\hat{\beta})$ for the linear model, where $E(r)=\varphi$, $E(\hat{\alpha})=\alpha$ and $E(\hat{\beta})=\beta$, and (r) represent the correlation coefficient (Table 2).

Table 2. Confidence intervals (95%) of α , β and φ on linear model calculated by Fisher's analysis.

-95%		Parameters		+95%
3321.8	$\leq$	α	$\leq$	3358.5
581.4	$\leq$	β	$\leq$	805.8
0.596	$\leq$	φ	$\leq$	0.999

Fig. 6 Linear model analysis on integral fading (IF) and four reference DNA size. The linear equation was estimated by Fisher's analysis. Discontinues line plotted represents standard error calculated. Spermatozoa: EZ, tilapia; EZR, red abalone; EZAZ, blue abalone; EZAM, yellow abalone. Haemocytes: HemAZ, red abalone; Heme, red abalone; HemAM, yellow abalone. Erythrocytes: ERT, tilapia; ERTTr, rainbow trout.

4. Discussion

Although the accurate measurement of genome sizes is not simple, it is enough practical and theoretical importance to justify the effort. Recent advances in computing and image analysis technology has allowed obtain a rapid and reliable estimation of genome size⁵. Image analysis densitometry has been accepted as an accurate means of quantifying DNA for diverse applications^{6, 17}, and its broader utility in the measurement of both plant and animal genome⁵. However, the

disadvantage of this methodology resides basically in two aspects; the staining protocol and its quantifying in order to obtain an accurate measurement of genome size.

The Feulgen reaction utilized for image analysis densitometry is an ordered series of chemical reactions and for this reason it is possible to rely on the simple premise that the amount of bounded stain is directly proportional to the amount of nuclear DNA present. Nevertheless, the protocol has been modified frequently and substantially since its early development, but the basic components have not been altered. The major modification has been around the condition of hydrolysis and 'basic fuchsin' type used for formulation of Schiff's reagent⁵. The Feulgen reaction requires a hard hydrolysis in relatively weak acid, chloride acid 1 N at 60°C (hot hydrolysis) or in strong acid to 5 N at 20-30°C (cold hydrolysis). The suggested time for hydrolysis has also varied greatly, and must be altered for each cellular type and fixing solution used¹⁸. These conditions for the Feulgen reaction can affect the efficacy of the stain and a standard must be treated with the sample to correct any variation on the staining procedure⁵.

Another complication in the estimation of genome size resides in that densitometric methodologies requires the quantifying of the absorbance, but it is not possible directly due that the absorbance is the lack of emitted light, and therefore represents non-information. The absorbance must be estimated indirectly by comparison of transmittance of light between the initial intensity of incident light in a Feulgen-stained nucleus and the transmitted light leaving it. The problem of this approach is to determine the accurate intensity on the field or sample, independently of the effect of field density (nuclei per field), age of slides, cell type, etc⁵.

4.1 DNA-Fluorescence fading and DNA nuclear size

In fluorescence observation by conventional fluorescence microscopy, the retardation of fluorescence fading, the high initial intensity of images, and low background noise are important factors for obtaining accurate images¹⁴. DAPI stain is the most widely used fluorochrome for stain DNA nuclear in fluorescence microscopy. However, the fluorescence of DAPI is rapidly lost when it is exposure to excitation by U.V light, and especially under optimal condition for observation: under illumination at the wavelength of maximal absorbance of the fluorochrome and with objective lenses of a high numerical aperture¹⁹. The photochemical process underlying the fluorescence decay has not yet been fully explained, although some theories suggest the involvement of oxygen, triplet states, and protein denaturalizations^{20, 21}.

The analysis of genome size by microfluometry methods uses fluorescent dyes or fluorochromes. This fluorochromes bind stoichiometrically to the DNA of each cell. Consequently, the amount of fluorescence is proportional to the total amount of DNA in each cell. The loss or addition of DNA in a population of cells is thus detectable¹². A variety of DNA-fluorescent stain has been currently used and may be classified into two groups; fluorochromes that intercalate between base pairs within the phosphate backbone of double-stranded DNA, and DNA-specific stains that bind to guanine-cytosine or adenine-thymidine within DNA. In the last type we found the 4'-6 diamidino-2-phenylindole (DAPI). The disadvantage of the microfluometry methods is that it is not possible to exposure for long times the DNA-stained samples, because the fluorescence fading can affect the quantifying of fluorescence, and therefore the DNA content.

According to ours results, we found evidence that the decrease in fluorescence intensity is a function of the U.V light exposure time, because in all nuclear type analyzed the DAPI-fluorescence is rapidly lost after exposure to U.V light. But, what is it the relationship between the fluorescence fading and the genome size?, to ascertain this, we decided to quantify the fluorescence fading by means of the estimation of the area under the graph during fading period, for this relation we named *integral fading*, because the area under the graph represents a integral function. The integral fading (IF) calculated for each species, demonstrated that are different for each nuclear type; in spermatozoa and red blood cells of *Oreochromis mossambicus*, IF = 1416.58 and IF = 2825.90 respectively, and in spermatozoa of *Haliotis rufescens*, IF = 3080.28 and in red blood cells of *Oncorhynchus mykiss*, IF = 9642.80. Additionally, we calculated the number of nuclei (sample size) required to estimate the representative IF value, and in terms of the variance and mean IF, it is possible have an accurate IF value around 150 nuclei. These IF values have a linear relationship with the genome size reported for this species ($r = 0.99$).

The present method for genome size estimation analyzed the information of the fluorescence fading by image processing, gives solution to quantify fluorescence in only one time, because this measurement discard the fact that

fluorescence decrease in time function. We propose to measure during the fading period, to obtain the fading behavior for the nucleus stained with DNA-stain. According to IF values they were directly proportional with respect to genome size, and therefore we propose two ways for calculating the genome size uses IF values (equations 1 and 2).

The advantage of the method proposed is that the DAPI-stain for DNA nuclear is a very simple stain method, and therefore easy for standardization. We have in mind to steps, first: to improve this methodology for genome size estimation it is necessary to build an accurate linear model to ascertain an accuracy and quantitative method that may allow comparatives analysis of genome size; and second: to use paralleling compute in order to reduce time-consuming when we are analyzing many samples. Parallel calculation consists of using computers with more of a processor reducing the run time of the programs. Therefore, parallel calculation is able to reduce the run time of the program to $1/N$, where N is the number of used processors.

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