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Standardisation of instrumentation in plant DNA image cytometry

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DNA image cytometry is a relatively new technique for densitometric measurement of nuclear DNA content, which has only rarely been used in botany and thus no methodological standards exist for this method to be applied to the measurement of plant material. In the present paper we address several problems related to standardisation of DNA image cytometry, such as stability of the measuring system, linearity of optical density measurements, correction of uneven illumination of the field of view, and uniformity of integrated optical density measurement over the entire field of view. Furthermore, image processing procedures are described for mitigation of the effects of electronic noise (image averaging) and for densitometric calibration of the measuring system. We have developed a macro for plant DNA image cytometry, using a general image analysis software package. The described quality control procedures, adopted from international medical standards for diagnostic DNA image cytometry, were used during software development to test performance of our measurement system. Until a specific botanical consensus is reached, we recommend that the quality control standards for instrumentation described in the present paper are considered when DNA image cytometry is used for measurement of plant genome size as well as for any other image analysis-related densitometric measurement based on light microscopy.

Key words: DNA image cytometry, standardisation, instrumentation, nuclear DNA content, plant material

Abbreviations: CV – coefficient of variation, IOD – integrated optical density, ND filter – neutral density filter, OD – optical density

Introduction

The international meeting on genome size in angiosperms held at The Royal Botanic Gardens Kew in 1997 addressed aspects of best practice for measurement of genome size, including standardisation of measurement protocols (BENNETT 1998). Several burning questions concerning the methodology for nuclear DNA content measurement in plants, however, still remain unresolved.

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In general, two densitometric methods are used to evaluate plant genome size: photometric cytometry and image cytometry (BENNETT and LEITCH 1995, BENNETT and LEITCH 1997, BENNETT et al. 2000). In both methods, DNA is usually quantitatively stained with the Feulgen reaction. The intensity of staining in the nuclei is measured according to classical protocols with a cytophotometer or, more recently, with DNA image cytometry (BÖCKING et al. 1995) using an image analysis system (a microscope linked to a personal computer via a camera (JARVIS 1986, SÁNCHEZ et al. 1990, CHIECO et al. 1994, BÖCKING et al. 1995). In this case, an image analysis software package is used to process the grabbed images and calculate the integrated optical density of the nuclei. Using a standard species with a known genome size (nuclear DNA amount expressed as the C-value), the arbitrary units of the integrated optical density (IOD) measurement are converted to picogram (pg) DNA.

With the rapid development of digital imaging technologies, image analysis systems are bound to replace classical cytophotometers. Although DNA image cytometry is routinely used in medical science for diagnostic purposes, mostly to evaluate the ploidy status of a cell population, this method has only rarely been applied in botany for measurement of nuclear DNA amount (TEMSCH et al. 1998, TEMSCH et al. 1999, DIMITROVA et al. 1999, YU et al. 1999, VILHAR et al. 2001a, b).

Whereas medical scientists have recently published a number of papers aiming at reaching an international consensus on the methodology of nuclear DNA content measurements (BÖCKING et al. 1995, PUECH and GIROUD 1999, ORMEROD et al. 1998, and refs. therein), few botanists have comprehensively addressed these questions (for example, BENNETT and SMITH 1976, DOLEŽEL et al. 1998, JOHNSTON et al. 1999, GREILHUBER and TEMSCH 2001, VILHAR et al. 2001a). To some extent, image cytometry has been compared to other methods used for plant genome size measurement (TEMSCH et al. 1998, TEMSCH et al. 1999, DIMITROVA et al. 1999, VILHAR et al. 2001a), but methodological standards for quality control of instrumentation have not yet been discussed in botanical literature.

Several DNA image cytometry software packages designed for routine medical diagnostics are commercially available. These image analysis systems can also be used for measurements of plant material (VILHAR et al. 2001a). In our laboratory, we developed a DNA image cytometry macro, based on a general image analysis package. We have recently demonstrated that such user-defined macros can give results comparable to those obtained with medical software packages, flow cytometry and photometric cytometry (VILHAR et al. 2001a).

In the present paper, we address several problems that need to be solved appropriately for accurate densitometric measurement of nuclear DNA content with an image analysis system. The performance of our measurement system was evaluated against set or proposed international medical standards (BÖCKING et al. 1995, PUECH and GIROUD 1999) and the presented results may serve as an example of standardisation procedures. Where applicable, plant nuclei were used at different stages of software development and testing. We propose that the medical methodological standards for DNA image cytometry be adopted for measurement of plant material until such time as international botanical standards are agreed.

Materials and methods

The image analysis system

A light microscope (Axioskop MOT, Carl Zeiss) was connected to a personal computer via a colour CCD camera (Sony DXC–950P). A green filter was inserted in the light path for densitometric measurements (band pass interference filter, 540 nm, width at half-peak transmission 90 nm) and Köhler illumination was used, with objective magnification 40 × (Plan-Neofluar, Carl Zeiss). Grey images were grabbed using the green camera channel with the frame grabber Matrox Meteor (image size 760 × 560 pixels) and processed using macros based on the software package KS 400 version 3.0 (Carl Zeiss Vision).

General conditions for the quality control procedures

For all quality control procedures, the microscope and image acquisition settings were the same as those used for genome size measurements (VILHAR et al. 2001a). The image of nuclei was focused, then the field of view was moved to an empty field, and the image was grabbed. A set of neutral density (ND) filters with known transmission (T = 1.5, 6, 9, 25, 40, 45 and 80%) was used where indicated. The theoretical optical density (OD) value for each filter was calculated from T as OD = log (1/T). The settings of the system were not changed during the whole measurement session. All the quality evaluation procedures were repeated several times with similar results.

Evaluation of instrumentation warming-up period

The mean grey value of the entire field of view was recorded every 2 min after the system had been switched on (PIRARD et al. 1999). The measured grey values were plotted against time.

Evaluation of electronic noise and drift over time

During both quality control procedures, an ND filter (T = 0.4) was inserted in the light path in order to obtain measurements in the intermediate grey value scale. For evaluation of electronic noise, the grey value of the pixel in the centre of each of the five fields of the image shown in Figure 1A was measured every 2 min during 1 h (PUECH and GIROUD 1999). In addition, a set of 100 images was grabbed at the maximum speed of the measuring system and the grey values of the same pixels were recorded on each image. For evaluation of drift over time, the mean grey value of the whole image was measured every 2 min during 8 h (PUECH and GIROUD 1999). The coefficient of variation (CV) of each measurement series was calculated as the standard deviation of grey values divided by the mean grey value (PUECH and GIROUD 1999).

Evaluation of linearity

The OD of a 10 × 10 pixel field positioned in the centre of the image was measured using a set of ND filters. OD was measured at four additional 10 × 10 pixel fields positioned at midpoint between the centre and each of the corners of the image (Fig. 1A). The measured OD values were compared to the theoretical OD of each filter. The slope of the linear

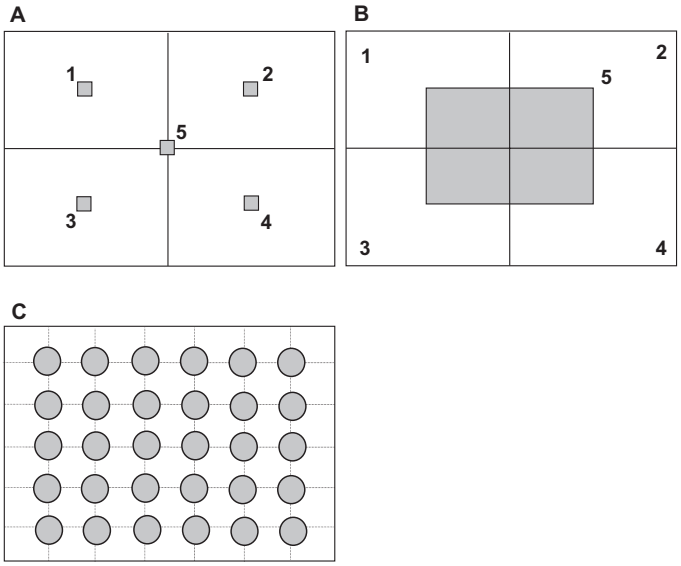

Fig. 1. Definition of measurement fields used for quality control tests. The outline represents the whole image (760 × 570 pixels). A – evaluation of electronic noise and linearity (10 × 10 pixel fields); B – evaluation of shading; C – 30 positions of the nucleus (shown as circles) for evaluation of uniformity of measurement over the entire field of view. For evaluation of electronic noise, the grey value of the pixel in the centre of each of the five fields shown in A was measured.

regression (theoretical vs. measured OD) and the coefficient of determination (r^2) were calculated for each field (PUECH and GIROUD 1999). Finally, the coefficient of variation of the five slopes was calculated (PUECH and GIROUD 1999).

Evaluation of shading

The image was divided into five fields according to PUECH and GIROUD (1999) as shown in Figure 1B. The OD of the five fields of the image was measured and evaluated with the same procedures as those described for linearity evaluation.

Evaluation of measurement uniformity over the entire field of view

Root tips of *Allium cepa* L. were fixed, stained with the Feulgen reaction and squashed to obtain a monolayer of cells on a microscope slide as described in VILHAR et al. (2001a). The same nucleus was placed at 30 different positions in the field of view (a grid with six horizontal and five vertical lines, Fig. 1C), and the integrated optical density (IOD) of the nucleus was measured. The coefficient of variation was calculated for the 30 IOD values (BÖCKING et al. 1995).

Results and discussion

The microscope can be converted into a precise densitometric instrument, given that every step of the measurement, from image acquisition to calculation of IOD, is optimised and standardised (BÖCKING et al. 1995, OBERHOLZER et al. 1996, PUECH and GIROUD 1999, PIRARD et al. 1999). In the present study, we address the following topics that need to be thoroughly examined in DNA image cytometry:

1. evaluation of stability of the measuring system (system warming up period, electronic noise, drift over time) and procedures to reduce the effects of electronic noise
2. evaluation of linearity
3. evaluation of uneven illumination of the field of view (shading)
4. densitometric calibration of the image analysis system
5. the procedure for calculation of nuclear IOD
6. uniformity of IOD measurement over the entire field of view

The general aim of DNA image cytometry is to measure the true grey value of each pixel as defined by GOLDSTEIN (1970) for photometric cytometry. The true grey value is the grey value related to the optical properties of the measured object, whereas the apparent grey value corresponds to the grey value of the same pixel in the original grabbed image. Image processing procedures can be used to convert the apparent grey value to the true grey value. Thus, a linear relationship is established between grey values and transmission, and the precision of this relationship can be tested with quality control standards for instrumentation. Measurement of the true grey value is prerequisite for accurate OD calculation.

Evaluation of system stability

The test of the stability of the system comprised the warming-up test (PIRARD et al. 1999) and a separate evaluation of electronic noise and drift over time (PUECH and GIROUD 1999).

Warming up

At the beginning of each measurement session, the system needs to be allowed sufficient time to warm up. In the absence of a cooling device, the camera slowly warms up and the rising temperature provokes an undesired electron excitation leading to fluctuations of the recorded grey values under constant illumination (PIRARD et al. 1999). Figure 2 shows the time course of grey value measurements after the system had been switched on. The data indicate that the response of our system stabilises after approx. 90 min. Similar warming up times have been reported for other types of cameras (PIRARD et al. 1999). The same test was used with different ND filters inserted to record different grey values with similar results (data not shown). The 90 min warming-up period was regularly used in our experiments.

Electronic noise

Electronic noise leads to scattering of the electrical signal coming out of a photodiode submitted to a constant amount of photons. Several major sources of electronic noise can be identified in a CCD, such as photon noise, dark current noise and digitisation noise (CHIECO et al. 1994, PIRARD et al. 1999). The effects of electronic noise are illustrated in Figure 3.

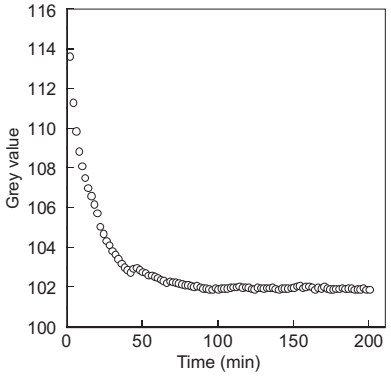

Fig. 2. Stability of the measuring system during the warming-up period. Mean grey value of the whole field of view at various times after switching the system on is shown (green channel of the camera). The ND filter with T = 40% was used to record intermediate values on the grey value scale.

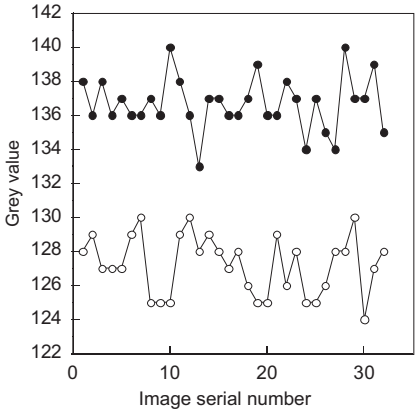

Fig. 3. Electronic noise of the measuring system. ○ – the grey value of a single pixel measured from a series of 32 images grabbed at maximum system speed; ● – the grey value of a single pixel measured every 2 min over 1 h. The number on the x-axis indicates the serial number of the image for each measurement.

In the standard electronic noise test, the grey values of single pixels were measured at 2 min intervals over 1 h (PUECH and GIROUD 1999). For the system to qualify for DNA image cytometry, the CV of grey values for a single pixel should not exceed 2% (PUECH and GIROUD 1999). Using our system, the CV was about 1.6% (Tab. 1).

The noise of the electronic system was further examined by grabbing a set of 100 images of the same field of view at the maximum speed of the system (Tab. 1). Such a rapid measurement reveals fluctuations in measurements due to electronic noise (PIRARD et al. 1999), while eliminating the effects of drift over time that may occur during the one hour measuring period. For this test, grey values of single pixels at the centre of the five fields shown in Figure 1A were recorded with different ND filters to give different values on the

Tab. 1. Evaluation of electronic noise and drift over time. Noise (measurement every 2 min over 1 h): the grey value of the central pixel of the image was measured every 2 min over 1 h (N = 30). Noise (measurement of a series of 100 images): the grey value of the central pixel of the image was measured in a series of 100 images taken at maximum system speed (N = 100); central pixel – measurements represent variation in the 100 images; averaged images – the grey values of the central pixel were averaged for 32 serial images (images 1–32, 33–64, 65–96) to give three central pixel values – the variation of these three values is shown (N = 3). Drift over time: the mean grey value of the entire field of view was recorded every 2 min over 8 h (N = 240). The mean grey value and the coefficient of variation (CV expressed in %) are shown. A set of ND filters (T = 1.5, 6, 9, 25, 40, 45, 80%) was used as indicated in the table legend. The standards are quoted from PUECH and GIROUD (1999).

	Filter T (%)								
	100*	80	45	40	25	9	6	1.5	0**
				Noise (measurement every 2 min over 1 h)					
Standard				*CV<2%*					
Central pixel									
mean grey value				136.7	103.1				
CV (%)				1.6	1.6				
				Noise (measurement of a series of 100 images)					
Central pixel									
mean grey value	243.8	216.1	141.9	127.2	92.0	31.8	20.1	4.4	1.1
CV (%)	0.6	0.7	1.3	1.4	1.9	6.6	9.9	22.2	27.4
Averaged images									
mean grey value	243.7	216.1	141.9	127.1	92.0	31.8	20.1	4.4	1.1
CV (%)	0.1	0.0	0.1	0.1	0.3	0.9	2.8	2.2	4.5
				Drift over time					
Standard				*CV<2%*					
mean grey value				121.0					
CV (%)				0.3					

* An empty field of view on the slide was measured;
** The black image (blocked light path) was measured.

grey value scale (Tab. 1). The single pixel measurement showed an increasing CV of the grey values with increasing OD. Similar results were obtained for all five pixels measured (only data for the central pixel of the image are shown in Table 1).

The CV of the grey values was markedly reduced when the average grey value was measured for a series of 32 images (Tab. 1). In this averaging procedure, images number 1–32, 33–64 and 65–96 were used to get the mean grey value of the pixel. The CV of the mean grey values for the three averaged images was calculated (Tab. 1). Compared to the protocol using one grabbed image, with the averaging procedure the CVs of the mean grey values were relatively low even for pixels with a high OD.

For precise densitometric purposes, every care should be taken to mitigate the influence of electronic noise on the measurement. According to PIRARD et al. (1999), a fluctuation range of 2 to 8 grey values for single pixels is not uncommon in standard video cameras. This problem is particularly acute in the dark range of the greyscale (Tab. 1), where the OD

curve plotted against the available grey values is very steep. However, the effects of electronic noise can be reduced by taking the arithmetic average of a sequence of images (Tab. 1, PIRARD et al. 1999, CHIECO et al. 1994). In our densitometric measurements, the image averaging procedure is routinely applied.

A lower electronic noise as well as a higher resolution can be achieved with replacement of a video camera with a digital camera (John C. Russ, pers. comm.). However, digital cameras are a relatively new and expensive technology, some software packages have not yet been converted to the higher resolution range, and video cameras are presently still frequently purchased to support microscope-based image analysis.

The halogen lamp used to illuminate the field of view in a microscope is a potential source of electronic noise that should be considered in image densitometry. While it gives relatively stable illumination at the middle of its life time, a new halogen lamp and, in particular, a lamp approaching the end of its life time can flicker, thus substantially contributing to the overall electronic noise of the measurement system. Hence, regular checking of stability of measurement is recommended.

Drift over time

According to the standards for the drift over time evaluation, the CV of the mean grey value of the entire field of view should not exceed 1% during an 8 h test period (PUECH and GIROUD 1999). For our system, the CV was 0.3% at the mean grey value 121.0 (maximum 121.9, minimum 120.4; Tab. 1).

Since the temperature affects the sensitivity of the camera (Fig. 2), temperature fluctuations during measuring session (such as variation in ambient temperature) may lead to drift of the instrument and should therefore be avoided.

Evaluation of linearity

Theoretically, photodiodes have a linear response with respect to incident light intensity (transmission). Nevertheless, this has to be verified experimentally for each individual camera (PIRARD et al. 1999, CHIECO et al. 1994, PUECH and GIROUD 1999).

For the evaluation of linearity, five fields (10×10 pixels) were defined on the image as shown in figure 1A. OD was measured for each field using a set of ND filters. According to the medical standards, the slope of the regression line plotted over a series of measurements with different ND filters for one field should fall between 0.9 and 1.1. In addition, the coefficient of determination (r^2) should be higher than 0.99 for the image analysis system to qualify for precise DNA measurement, and the standard error of estimate (SE_{yx}, expressed in %) lower than 5% (PUECH and GIROUD 1999).

With our system, the average regression slope was 1.082 (lowest 1.078, highest 1.084), r^2 was 0.993 and SE_{yx} was between 6.0 and 6.3% (Tab. 2). Hence, the system showed linearity that was within the standard limits, except for a slightly too high SE_{yx}. This error was corrected for with densitometric calibration (see below).

Evaluation of shading

In image cytometry, the measured area covers a large portion of the microscopic field of view. Optical lenses generally pass more light in the centre, thus causing a shadow effect

(shading) at the edges of the image (CHIECO et al. 1994). Illumination defects may also be caused by camera properties (OBERHOLZER et al. 1996).

The effect of shading on OD measurement was tested with a procedure similar to that for evaluation of linearity, except that the measured fields of the image were defined as shown in Figure 1B (PUECH and GIROUD 1999).

For medical applications, the standard for coefficient of variation of OD regression slopes for the five fields measured with different ND filters is CV < 2% (PUECH and GIROUD 1999). The CV of regression line slopes in the five fields measured with our system was 0.2% (Tab. 2), thus falling well within the limits of acceptable values.

Shading correction procedures may be used during image processing to decrease shading effects. Such procedures are listed among standard methods used to measure nuclear DNA content for medical purposes (BÖCKING et al. 1995). Several algorithms available for shading correction procedures are applicable to densitometry (CHIECO et al. 1994, PIRARD et al. 1999, OBERHOLZER et al. 1996). The procedure described by PIRARD et al. (1999) was applied during measurement of nuclear DNA content in nine plant species, with satisfactory results (VILHAR et al. 2001a). Nevertheless, shading correction involves the process of subtracting or dividing images by another (depending on whether the camera has a linear or logarithmic response), during which some information contained in the original image gets lost (RUSS 1998). Hence, all practical steps should be taken to make illumination of the grabbed image uniform before resorting to processing methods (OBERHOLZER et al. 1996).

Tab. 2. Evaluation of linearity and shading. A set of ND filters (T = 1.5, 6, 9, 25, 40, 45, 80%, blank field of view T=100%) was used to plot a regression line (theoretical OD vs. measured OD; y = kx + n) for each of the fields shown in Figure 1A for linearity test and Figure 1B for shading test. The images were processed with two different procedures: without densitometric calibration (the gamma correction of the camera switched off) and with densitometric calibration (the gamma correction of the camera switched on). The same system settings were used for both procedures. r^2 – coefficient of determination, SE_{yx} – standard error of estimate, CV_k – coefficient of variation of regression slopes. The standards are quoted from PUECH and GIROUD (1999).

Procedure	without densitometric calibration (gamma correction off)					with densitometric calibration (gamma correction on)				
Test	Slope (k)	Const. (n)	r^2	SE_{yx} (%)	CV_k (%)	Slope (k)	Const. (n)	r^2	SE_{yx} (%)	CV_k (%)
					Linearity					
Standard	*0.9-1.1*		*>0.990*	*<5%*		*0.9-1.1*		*>0.990*	*<5%*	
mean values (for five fields)	1.08		0.993	6.2	0.3	0.99		0.999	1.5	0.1
maximum slope	*1.08*	*0.007*	*0.093*	*6.0*		*0.99*	*0.004*	*0.999*	*1.6*	
minimum slope	*1.08*	*0.008*	*0.993*	*6.3*		*0.99*	*0.002*	*1.000*	*1.3*	
					Shading					
Standard					*<2%*					*<2%*
mean values (for five fields)	1.08		0.993	6.1	0.2	0.99		0.999	1.5	0.1
maximum slope	*1.08*	*0.007*	*0.994*	*5.9*		*0.99*	*0.003*	*0.999*	*1.5*	
minimum slope	*1.08*	*0.007*	*0.993*	*6.2*		*0.99*	*0.002*	*1.000*	*1.4*	

Densitometric calibration

As shown in Table 2, our system showed relatively good linearity with the settings used in a typical genome size experiment with the gamma correction of the camera switched off. However, in some cases these settings may be changed for a better resolution of very weakly or strongly stained nuclei. For example, the use of gamma correction of the camera provides additional grey values at high absorbance values, thus extending the dynamic range of the system (Fig. 4; CHIECO et al. 1994). This effect proved beneficial for measurement of darkly stained nuclei, such as those found in *Allium cepa*. It is important to note that in some cameras, when the gamma correction is applied, some automatic functions of the camera may also be switched on, hence the relationship between grey values and transmission should be thoroughly tested before the gamma correction is used in densitometric measurements (John C. Russ, pers. comm.).

Any departures from linearity can be adequately corrected for during image processing, when a densitometric calibration procedure is applied prior to OD measurement. The grey values for a set of ND filters are recorded with the procedure used for linearity evaluation (measurement of the central 10 × 10 pixel field or the whole image). A calibration curve is plotted as shown in Figure 4 and the resulting regression curve used to generate a look-up table which is then used to transform the images prior to densitometric measurements (CHIECO et al. 1994). With this procedure, new grey values are assigned to each pixel and the linearity of the system is recovered (Fig. 4). With the camera gamma correction switched on, a two or three degree polynomial function representing the relationship between transmission and grey values is sufficient to obtain linearity for OD values 0 – 1.6 (CHIECO et al. 1994).

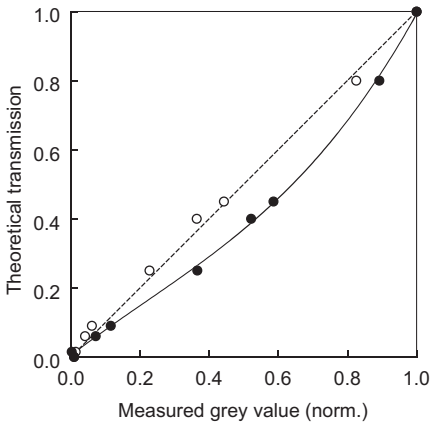

Fig. 4. Densitometric calibration curve for genome size measurement. In order to get calibration points, the mean grey values of the entire field of view (empty field of view on the microscope slide) were measured using a set of ND filters with known transmission values. ○ – measured transmission values with the gamma correction switched off; ------ – the ideal linear relationship between theoretical and measured transmission values; ● – measured transmission values with the gamma correction switched on; —— – the polynomial regression curve used to calibrate the system with the gamma correction switched on (equation of the regression curve: $y = 0.597x^3 – 0.380x^2 + 0.770x + 0.005$; $r^2 = 0.999$). The grey values were normalised to assign value 1.0 to the mean grey value of an empty field of view.

The quality control procedures for linearity and shading evaluation were performed again under the same conditions as described above for the uncalibrated system, but with images subjected to the densitometric calibration procedure prior to OD measurement. In this case, the gamma correction of the camera was switched on.

In the linearity evaluation test, the uncalibrated and the calibrated system gave similar results (Tab. 2). The high SE_{yx} observed for the uncalibrated system was corrected when densitometric calibration was applied (Tab. 2), and the CV of the five slopes decreased from 0.3% to 0.1%. In addition, the regression line passed closer to the zero intercept when the densitometric calibration procedure was applied, and the coefficient of determination r^2 increased from 0.993 to 0.999 (Tab. 2). In the shading evaluation test, the resulting CV of the regression slopes for the five image fields improved from 0.2% for the uncalibrated to 0.1% for the calibrated system (Tab. 2).

In general, an appropriate densitometric calibration that accounts for the use of the gamma correction yields results comparable to those of the uncalibrated system with the gamma correction switched off in terms of linearity and shading (Tabs. 2 and 3). Furthermore, good results were obtained from densitometric measurements of plant nuclei when the gamma correction was switched on and the system was densitometrically calibrated (data not shown).

According to PUECH and GIROUD (1999), colour cameras have a poor performance compared to black-and-white cameras. Our system using a colour camera has given satisfactory results during the quality control tests, indicating that a colour camera can show a good performance, given that only one channel of a 3CCD camera is used (John C. Russ, pers. comm.). Nevertheless, it is important to examine different settings of the camera and the frame grabber to achieve optimal quality of the grabbed image (PIRARD et al. 1999).

For quality control procedures and densitometric calibration, a reference slide with mounted ND filters can be used instead of a set of ND filters (PUECH and GIROUD 1999). However, the reference slide can currently only be ordered in batches over 30 slides, and each slide is relatively expensive. Alternatively, ND filters can be cut and mounted on a slide (PUECH and GIROUD 1999). During the presented tests, ND filters were inserted in the light path, hence ND shading was applied over a true measuring field, with the microscope slide inserted but shifted to an empty field of view.

Calculation of IOD

The following equation was used to calculate IOD for each nucleus, according to the Lambert-Beer law:

$$IOD_n = \Sigma_i OD_{ni} = \Sigma_i [\log (GV_{bi} / GV_{ni})]$$

where IOD_n is the IOD of the nucleus, i is the number of pixels in the nucleus, $\Sigma_i OD_{ni}$ is the sum of ODs calculated for i individual pixels in the nucleus, GV_{ni} is the grey value of the pixel i in the nucleus, GV_b is the grey value of pixel i on an image of background (empty field of view) and Σ_i denotes the sum of values calculated for individual pixels.

With this procedure, the IOD of the nucleus represents the sum of ODs calculated for each pixel within the nucleus. The calculation of IOD must be based on individual pixel grey values and not on the mean grey value of the nucleus, because the sum of the loga-

rithms of transmission values does not equal the logarithm of the mean transmission value multiplied by the number of pixels.

The DNA image cytometry system presented in this study has a resolution of approx. 24 pixels per μm^2. The cytophotometers typically give a resolution of 4 measurements over one μm^2 of the object when a 0.5 μm scanning step is used. Thus, the resolution of our instrument, which represents a typical system for DNA image cytometry, is higher than in photometric cytometry.

The Lambert-Beer law is valid only when photometric readings are taken with monochromatic light, hence filters are applied during DNA image cytometry to limit the wavelength range used for the measurement. The absorption maximum for Feulgen-stained nuclear DNA lies at 565 nm (BEDI and GOLDSTEIN 1976). The medical standards recommend application of a 560 ± 10 nm interference filter (BÖCKING et al. 1995, CHIECO et al. 1994), although band-pass gelatine filters can give similar results (CHIECO et al. 1994).

Evaluation of uniformity of IOD measurements over the entire field of view

According to medical DNA image cytometry standards, for measurement of one diploid nucleus at 30 different sites of the digitised image a CV of IOD values lower than 3% is acceptable (BÖCKING et al. 1995). With our system, the CV of IOD values for 30 positions (Fig. 1C) of Feulgen-stained *Allium cepa* nuclei was below 1% (Tab. 3).

Specific problems of nuclear DNA content measurement in plant material

In the present study, we illustrate one of the possible approaches to the application of DNA image cytometry to plant nuclear DNA measurement, that is, the development of user-defined macros for densitometric purposes based on a general image analysis software package. Arguably it is less cumbersome to purchase one of the commercially sold medical DNA image cytometry software packages, keeping in mind that these packages are relatively expensive, can do little else in terms of image analysis than nuclear DNA amount measurement and that they often contain medical diagnostic tools which are useless in botany. On the other hand, a general programmable image analysis package can be adapted for

Tab. 3. Evaluation of uniformity of measurement over the entire field of view with nuclei placed at 30 different positions on the image. The 30 positions of the nucleus (*Allium cepa*) on the image are shown in Figure 1C. The images were processed with two different procedures: without densitometric calibration (the gamma correction of the camera switched off) and with densitometric calibration (the gamma correction of the camera switched on). The measured IOD is shown as the mean value ± standard error of the mean (SEM). The standard is quoted from BÖCKING et al. (1995).

Procedure	without densitometric calibration (gamma correction off)			with densitometric calibration (gamma correction on)		
Nucleus	Size (μm^2)	IOD (a.u.) mean ± SEM	CV (%)	Size (μm^2)	IOD (a.u.) mean ± SEM	CV (%)
Standard			*<3%*			*<3%*
large	287	3533 ± 3	0.5	256	3138 ± 3	0.6
small	118	1834 ± 2	0.5	113	1581 ± 1	0.5

applications other than nuclear DNA measurements, and can thus serve as an effective versatile instrument in a laboratory involved predominantly in research rather than in routine measurements.

Measurement of nuclear DNA amount over a range of plant species with variable genome sizes requires a wide dynamic range of the measuring system. During development of the DNA image cytometry macro presented in this study, we have tested performance of our measurement system in nine plant species over a 100–fold genome size range (2C nuclear DNA content approx. 0.3 – 33 pg DNA; VILHAR et al. 2001a). The results obtained with DNA image cytometry were compared to those recorded with accepted methods for plant genome size measurement, namely with flow cytometry and with photometric cytometry. Furthermore, stringent standards for quality control of the microscope slides were applied to test performance of the system (VILHAR et al. 2001a).

In applying densitometry to cytological preparations, a standardisation of the optical density (OD) of the background is implied (OBERHOLZER et al. 1996). The presence of the cell wall in plant cells may pose a problem in terms of accurate measurement of the background grey value. In our measurements, an empty field of view was used as the background reference (VILHAR et al. 2001a). The image of the background was reconstructed as the average of four images grabbed at different empty fields of view (32 averaged images each) on a slide. Alternatively, the background grey value can be determined locally for each individual nucleus as the mean grey value of a narrow band (a few pixels wide) surrounding the segmented nucleus. This approach is recommended in the medical standards (BÖCKING et al. 1995) and may further increase precision of the measurement.

Apart from the cell walls, other structures and substances specific to the plant cell may compromise the accuracy of DNA amount measurements. For example, starch grains may cause optical defects and different secondary metabolites interfere with the quantitative staining of DNA (GREILHUBER 1986). Hence, choice of appropriate tissue and rigorously controlled conditions of fixation and staining procedures (GREILHUBER and TEMSCH 2001) are prerequisite for measurement accuracy.

In our experience, when using DNA image cytometry in various plant species, it is relatively easy to get results which seem reliable, when in fact a systematical error may still be influencing the measurement, and hence biased nuclear DNA amount estimates are measured with relatively high precision (small variation of the measurement). Therefore, we recommend that quality control standards described in the present paper and in VILHAR et al. (2001a) are considered before any DNA image cytometry system is declared reliable in terms of plant nuclear DNA content measurement. Furthermore, we recommend that the DNA image cytometry system is tested over a wide range of plant genome sizes (same or similar to the species series described in VILHAR et al. 2001a) prior to its acceptance as an accurate tool for plant genome size measurement.

Conclusion

Several aspects of image acquisition and processing that need to be considered for accurate densitometry are not addressed in this paper, such as grey level working range adjustment, blooming and grey level clipping, segmentation (CHIECO et al. 1994, OBER-

HOLZER et al. 1996, PIRARD et al. 1999, PUECH and GIROUD 1999). Another procedure that may improve precision of IOD measurements is glare or stray light correction (GOLDSTEIN 1970, KINDERMANN and HILGERS 1994, PUECH and GIROUD 1999).

Given that reliability of DNA image cytometry in plant genome size measurement has only been systematically tested in two measurement systems (VILHAR et al. 2001a), the image analysis-related problems discussed in this paper should be addressed in other laboratories in order to standardise these methods applied to plant science. With application of the described standardisation procedures, it is possible to detect systematical errors in the measurement methods and to optimise DNA image cytometry. Only in this way can DNA image cytometry be accepted as a routine measurement method in botany. Furthermore, similar quality control procedures should be used when a microscope-based image analysis system is applied to any densitometric measurements (e.g. quantitative evaluation of immunostaining).

Finally, regardless of the method used for plant genome size measurement, an international consensus should be reached to provide standards for staining as well as measuring procedures. In our opinion, the medical standards for DNA image cytometry should be applied in botany until specific botanical standards are agreed.

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References

BEDI, K. S., GOLDSTEIN, D. J., 1976: Apparent anomalies in nuclear Feulgen-DNA contents. Role of systematic microdensitometric errors. J. Cell Biol. 71, 68–88.

BENNETT, M. D., 1998: Plant genome values: how much do we know? Proc. Natl. Acad. Sci. U.S.A. 95, 2011–2016.

BENNETT, M. D., BHANDOL, P., LEITCH, I. J., 2000: Nuclear DNA amounts in angiosperms and their modern uses – 807 new estimates. Ann. Bot. 86, 859–909.

BENNETT, M. D., LEITCH, I. J., 1995: Nuclear DNA amounts in angiosperms. Ann. Bot. 76, 113–176.

BENNETT, M. D., LEITCH, I. J., 1997: Nuclear DNA amounts in angiosperms – 583 new estimates. Ann. Bot. 80, 169–196.

BENNETT, M. D., SMITH, J. B., 1976: Nuclear DNA amounts in angiosperms. Philos. Trans. R. Soc. Lond. B Biol. Sci. 334, 309–345.

BÖCKING, A., GIROUD, F., REITH, A., 1995: Consensus report of the ESACP task force on standardisation of diagnostic DNA image cytometry. Anal. Cell. Pathol. 8, 67–74.

CHIECO, P., JONKER, A., MELCHIORRI, C., VANNI, G., VAN NOORDEN, C. J. F., 1994: A user's guide for avoiding errors in absorbance image cytometry: a review with original experimental observations. Histochem. J. 26, 1–19.

DIMITROVA, D., EBERT, I., GREILHUBER, J., KOZHUHAROV, S., 1999: Karyotype constancy and genome size variation in Bulgarian *Crepis foetida* s. l. (*Asteraceae*). Plant Syst. Evol. 217, 245–257.

DOLEŽEL, J., GREILHUBER, J., LUCRETTI, S., MEISTER, A., LYSÁK, M. A., NARDI, L., OBERMAYER, R., 1998: Plant genome size estimation by flow cytometry: inter-laboratory comparison. Ann. Bot. 82 (Suppl. A), 17–26.

GOLDSTEIN, D. J., 1970: Aspects of scanning microdensitometry. I. Stray light (glare). J. Microsc. 92, 1–16.

GREILHUBER, J., 1986: Severely distorted Feulgen-DNA amounts in *Pinus* (*Coniferophytina*) after non additive fixations as a result of self-tanning with vacuole contents. Can. J. Genet. Cytol. 28, 409–415.

GREILHUBER, J., TEMSCH, E. M., 2001: Feulgen densitometry: some observations relevant to best practice in quantitative nuclear DNA content determination. Acta Bot. Croat. 60, 285–298.

JARVIS, L. R., 1986: A microcomputer system for video image analysis and diagnostic microdensitometry. Anal. Quant. Cytol. Histol. 8, 201–209.

JOHNSTON, J. S., BENNETT, M. D., RAYBURN, A. L., GALBRAITH, D. W., PRICE, H. J., 1999: Reference standards for determination of DNA content of plant nuclei. Am. J. Bot. 86, 609–613.

KINDERMANN, D., HILGERS, C. H., 1994: Glare-correction in DNA image cytometry. Anal. Cell. Pathol. 6, 165–180.

OBERHOLZER, M., ÖSTREICHER, M., CHRISTEN, H., BRÜHLMANN, M., 1996: Methods in quantitative image analysis. Histochem. Cell Biol. 105, 333–355.

ORMEROD, M. G., TRIBUKAIT, B., GIARRETTI, W., 1998: Consensus report of the task force on standardisation of DNA flow cytometry in clinical pathology. Anal. Cell. Pathol. 17, 103–110.

PIRARD, E., LEBRUN, V., NIVART, J. F., 1999: Optimal acquisition of video images in reflected light microscopy. European Microscopy and Analysis 60, 9–11.

PUECH, M., GIROUD, F., 1999: Standardisation of DNA quantitation by image analysis: quality control of instrumentation. Cytometry 36, 11–17.

RUSS, J. C., 1998: The image processing handbook. 3rd edn. CRC Press, Boca Raton.

SÁNCHEZ, L., REGH, M., BIESTERFELD, S., CHATELAIN, R., BÖCKING, A., 1990: Performance of a TV image analysis system as a microdensitometer. Anal. Quant. Cytol. Histol. 12, 279–284.

TEMSCH, E. M., GREILHUBER, J., KRISAI, R., 1998: Genome size in *Sphagnum* (peat moss). Bot. Acta 111, 325–330.

TEMSCH, E. M., GREILHUBER, J., VOGLMAYR, H., KRISAI, R., 1999: Genomgrößen-Bestimmung bei *Sphagnum*: ein Methodenvergleich. In: Zechmeister HG, Hrsg. Bryologische Forschung in Österreich, Abhandlungen der Zoologisch-Botanischen Gesellschaft in Österreich 30, 159–167.

VILHAR, B., GREILHUBER, J., DOLENC KOCE, J., TEMSCH, E. M., DERMASTIA, M., 2001a: Plant genome size measurement with DNA image cytometry. Ann. Bot. 87,719–728.

VILHAR, B., VIDIC, T., JOGAN, N., DERMASTIA, M., 2001b: Genome size and the nucleolar number as estimators of ploidy level in *Dactylis glomerata* in the Slovenian Alps. Plant Syst. Evol., in press.

YU, F., DRISS-ECOLE, D., REMBUR, J., LEGUÉ, V., PERBAL, G., 1999: Effect of microgravity on the cell cycle in the lentil root. Physiol. Plant. 105, 171–178.