Estimation of nuclear DNA content in plants using flow cytometry

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Flow cytometry (FCM) using DNA-selective fluorochromes is now the prevailing method for the measurement of nuclear DNA content in plants. Ease of sample preparation and high sample throughput make it generally better suited than other methods such as Feulgen densitometry to estimate genome size, level of generative polyploidy, nuclear replication state and endopolyploidy (polysomaty). Here we present four protocols for sample preparation (suspensions of intact cell nuclei) and describe the analysis of nuclear DNA amounts using FCM. We consider the chemicals and equipment necessary, the measurement process, data analysis, and describe the most frequent problems encountered with plant material such as the interference of secondary metabolites. The purpose and requirement of internal and external standardization are discussed. The importance of using a correct terminology for DNA amounts and genome size is underlined, and its basic principles are explained.

INTRODUCTION

Flow cytometry (FCM) is an excellent method to analyze optical properties such as fluorescence and light scatter of microscopic particles in liquid suspension. Cells and cellular organelles are individually measured at high speed, typically 100–1,000/s, which facilitates representative sampling and detection of subpopulations. Some instruments can sort simultaneously up to four different subpopulations for further analysis and use. These features explain why FCM has become widespread in biomedical research and practice. Although less frequently employed in plant science and industry in the past, FCM is being increasingly used in a broad range of applications[1]. The estimation of nuclear DNA content is by far the most popular of these applications, with more than 800 publications to date[2].

Why nuclear DNA content?

The plant cell nucleus carries most of the hereditary material and, as such, has always been the subject of intensive studies. Estimation of DNA quantity (C-value)[3] in absolute units (DNA picograms and number of base pairs) has led to the discovery of more than 2,000-fold variation in the genome size in plants, with the smallest known genome of 63 Mbp found in *Genlisea margaretae* (Lentibulariaceae)[4] and the largest genome of 127 Gbp in the tetraploid *Fritillaria assyriaca* (Liliaceae)[5]. Although biological meaning of the variation remains obscure (cf. the C-value paradox/enigma)[6], a number of observations correlate genome size with cell cycle duration and cell size, and characters such as life cycle, weediness, threat of extinction, dry mass production, ecological requirements, phenology, seed mass and others (i.e., the nucleotypic theory)[7,8]. The availability of data on genome size is critical for many fields of research, including taxonomy and evolutionary changes[9]. Its knowledge is essential for planning gene cloning and genome sequencing projects[10].

Estimation of DNA content in relative units concerns equally important applications. The relationship between ploidy and nuclear DNA content makes the assay suitable for the determination of ploidy level, detection of mixoploidy and, under certain conditions, also aneuploidy. The applications range from taxonomy and population biology to breeding and quality control in seed production[11]. Simultaneous estimation of DNA content in seed embryo and endosperm makes it possible to determine the genetic origin of a seed, that is, establish its sexual or apomictic origin and gametic ploidy[12]. More specialized applications include the presence and extent of endopolyploidy[13,14] and the estimation of DNA base content[15,16]. As the nuclear DNA content undergoes characteristic changes during passage through the cell cycle, it is also possible to determine the position of a cell within the cell cycle[17]. Some studies have employed simultaneous analysis of nuclear DNA contents and the amounts of other cellular components such as RNA, proteins, secondary metabolites and/or expression of heterologous proteins[18,19]. Due to space limitations these methods are not included in this protocol, which deals exclusively with the estimation of nuclear DNA content.

Why FCM?

FCM has become the method of choice because it is convenient, fast and reliable. Sample preparation usually occupies only a few minutes and does not require expensive reagents. Analysis is rapid, and representative numbers of nuclei can be measured in a short time. Samples are typically prepared from only a few tens of milligrams of plant tissues; hence the method typically is non-destructive and suitable for the analysis of small individuals. An important advantage for ploidy estimation is that tissues containing dividing cells are not required. The fact that the nuclei are measured individually facilitates the analysis of samples with asynchronously dividing cells and the detection of subpopulations with different DNA contents. Nuclear DNA amounts can be analyzed with a high precision, with coefficients of variation (CV) of the DNA peaks generally ranging from 1 to 5% (ref. 2), while CVs of the means of replicate measurements are typically lower than 1% (personal observations).

The widespread adoption of DNA FCM was also stimulated by the availability of simple flow cytometers at affordable prices, sometimes capable of measuring only one fluorescence parameter. While static cytofluorometry was never widely adopted, Feulgen microspectrophotometry (or Feulgen densitometry) was the method of choice before it was supplanted by FCM, that is, until the late 1990s. While it measures individual nuclei, Feulgen densitometry offers similar advantages to FCM. Moreover, it is possible to select the nuclei for analysis and/or for visual inspection after analysis (but note that some current flow cytometers can also record images of measured particles). An advantage of Feulgen densitometry over FCM is that plant samples can be stored for prolonged periods before their processing and analysis (including the estimation of nuclear DNA content in absolute units). The major disadvantage of microspectrophotometry is that sample preparation is more laborious, and that the analysis is much slower. Similar advantages and disadvantages characterize image densitometry; however, until now this method has not been used widely in plant sciences, most probably because reliable software is not available commercially. Laser scanning cytometry combines the speed of FCM, its ability to measure multiple parameters and the possibility of visually inspecting measured particles[20]. However, due to the higher price of these instruments, this method will probably never be routinely used in laboratories where only DNA content is analyzed.

What are the options for sample preparation?

A sample for FCM is necessarily a liquid suspension of single intact particles. The use of intact cells for the estimation of DNA content is not recommended, because their size can approach or exceed the diameter of flow chamber orifices. Moreover, plant cells can disturb laminar flow due to their irregular shape and consequently interact with the excitation light beam differently, giving rise to variation in fluorescence and light scatter signals. Although this can be ameliorated by removing the cell walls to obtain spherical protoplasts, accurate analysis is still compromised by the unpredictably acentric position of the nuclei within the cell. Other drawbacks of measuring DNA within intact cells and protoplasts include the low permeability of the plasma membrane to most DNA fluorochromes, their nonspecific binding, autofluorescence coming from various pigments and interference of secondary metabolites with DNA staining. These difficulties can be partially overcome by treating protoplasts by a fixative (e.g., methanol) to permeabilize the plasma membrane and remove autofluorescent compounds such as chlorophyll. Provided the experiment does not require simultaneous analysis of nuclear DNA with other targets in cytoplasm, a viable option is to isolate intact nuclei.

How to isolate intact nuclei?

One way to isolate intact nuclei is to lyse protoplasts in a hypotonic buffer. In this manner, the nuclei are gently released from cells, and the procedure usually provides histograms of DNA content with a little background debris and DNA peaks having very low CVs[21]. However, protocols for protoplast isolation are laborious, and are available only for a limited number of species and for particular tissues at certain developmental stages. A universally accepted method for the preparation of nuclear suspensions for DNA FCM was developed by David W. Galbraith in his laboratory[22]. In this protocol, cell nuclei are released from a small amount of fresh plant tissue by chopping it with a sharp razor blade in an appropriate buffer solution. After filtration of the homogenate through a nylon mesh and addition of a DNA fluorochrome, the sample is ready for analysis. The method is rapid, convenient and suitable for many (though not all) plant species, organs and tissues.

Variations of Galbraith's protocol have been introduced, which differ in a way the sample is homogenized and by the composition of nuclear isolation buffers (see REAGENT SETUP for the composition of the four most popular buffers). Protocol modifications, including those suitable for nuclei isolation from recalcitrant plant tissues, such as pollen grains, are discussed elsewhere[9]. Recent options to release nuclei from various tissues include the so-called bead beating method, which involves shaking the tissues in a screw-capped tube containing zirconia/silica or glass beads (A.V. Roberts, personal communication). Release of nuclei from seed tissues has also been described by crushing between two sheets of fine sandpaper[12].

Although originally developed for fresh plant tissues, Galbraith's protocol seems suitable for the estimation of relative nuclear DNA content in dry herbarium and silica vouchers[23]. This advance expands the potential of DNA FCM to applications in taxonomy, population biology and ecology. A requirement for fresh samples evidently limits the application of DNA FCM in some research areas such as field botany, where samples are often collected in remote areas and cannot be immediately analyzed. Analysis of field-collected seeds is a useful option[24], but the results should be considered with caution as the origin of male gametes and hence the genotype of seeds is not known. Freezing would be a convenient way of storing plant samples. Although a few studies have demonstrated the feasibility of this approach[25], analysis of samples from frozen tissues usually results in lower resolution of histograms of DNA content and excessive background from debris (J.D. and J.S., unpublished observations).

Are fixed plant samples suitable?

The search for alternative methods of nuclei isolation coupled to the need to store samples before analysis has led to the development of methods in which fixed plant samples are analyzed. In fact, the first report on DNA content analysis in plants described the isolation of nuclei from acetic ethanol-fixed root tips, by digesting these tissues with pectinase followed by mechanical disintegration[26]. The potential of this approach is limited by the need to optimize the enzymatic treatment for different species and tissues, and by the difficulty of isolating single cells from differentiated tissues. A more universal method involved the mechanical release of intact nuclei from formaldehyde-fixed tissues[27]. Fixation increases nuclei yield and makes them suitable for immunofluorescent staining[28]. Fixation of isolated nuclei in 70 or 95% ethanol has also been used with some success[29]. While fixation can facilitate the storage of tissues and nuclei suspensions, it is known to modify chromatin structure and the accessibility of DNA to fluorochromes[30]. Hence, fixed plant samples cannot be recommended for the estimation of DNA content in absolute units until the reliability of such protocols is demonstrated.

And how to stain the nuclear DNA?

A fluorochrome used to estimate nuclear DNA content must bind selectively and stoichiometrically to DNA. A broad range of fluorochromes are known to specifically bind to DNA[31], but only

TABLE 1 | Basic characteristics of most popular DNA-selective fluorochromes used in plant flow cytometry.

Fluorochrome	Binding mode	Concentration used (μg ml^{-1})	Excitation wavelength (nm)[a]	Emission wavelength (nm)[a]	Suitable excitation source
Propidium iodide	Intercalating, no base preference	50–150	538	617	Green solid-state laser (532 nm), argon-ion laser (488 nm)[b]
Ethidium bromide	Intercalating, no base preference	50–150	523	603	Green solid-state laser (532 nm), argon-ion laser (488 nm)[b]
4′,6-diamidino-2-phenylindole	AT-selective	2–4	359	461	Arc lamp (UV band)
Hoechst 33258	AT-selective	2–4	352	455	Arc lamp (UV band)

[a]Excitation and emission spectra of DNA–fluorochrome complex were obtained from Invitrogen/Molecular probes. [b]Tunable argon ion lasers should be used at 514.5 nm.

a few are suitable for the quantification of DNA content in plants (**Table 1**). Ethidium bromide and propidium iodide (PI) intercalate into double-stranded DNA (and double-stranded RNA! (dsRNA)) without base-dependent bias, and thus are suitable for the estimation of DNA content in absolute units, provided the RNA is removed with RNase[32–34]. Fluorochromes binding preferentially to AT- and GC-rich regions of DNA are suitable for the estimation of DNA content in relative units when large differences in genomic base content are not expected, such as in autopolyploid series, and are necessary for DNA base content estimation. Out of them, DAPI (4′,6-diamidino-2-phenylindole) has been the most popular, as it provides DNA content histograms with high resolution, uses readily available excitation wavelengths and does not require RNase treatment.

How to interpret the measurements?

FCM records relative fluorescence intensities. Interpretation of results in terms of ploidy and genome size requires a reference standard (i.e., sample with known ploidy level and/or nuclear DNA content). The standardization may either be external or internal. The former involves successive analysis of the sample and the standard, whereas the latter involves simultaneous isolation, staining and analysis of nuclei both from sample and standard. A routine estimation of ploidy can be done using external standard although more precise ploidy analysis, and especially the detection of aneuploidy, requires internal standardization[35]. An individual belonging to the same plant species is usually employed in ploidy analysis, but animal species such as chicken red blood cells have been used, too. The estimation of DNA content in absolute units requires internal standardization, and animal standards are not recommended[34]. **Table 2** lists plant DNA standards with a range of genome sizes that are available free of charge from the corresponding author.

Is there a terminology to follow?

In order to communicate results, current terminology[3] should be used and attention paid to the life cycle-dependent nuclear phase of the material. For example, haplophasic tissues (with chromosome number n) dominate in bryophytes, whereas the majority of the tissues of tracheophytes are in diplophase (with a $2n$ chromosome number). An unreplicated nucleus of an individual in haplophase has a 1C DNA content, the letter 'C' standing for 'constant' and symbolizing the DNA amount of a 'holoploid genome' with chromosome number n. After DNA replication, the haplophasic nucleus has a 2C DNA content, the same as that of an unreplicated nucleus of an individual in diplophase. The degrees of endopolyploidy are usually best provided in C-levels. The DNA amount of a monoploid genome (i.e., monoploid genome size) with basic chromosome number x is given as its Cx-value. Thus, in somatic tissues of a hexaploid plant, such as bread wheat, *Triticum aestivum* ($2n = 6x = 42$), the 2C-value is equivalent to the 6Cx-value. The terms 'haploid', 'diploid', etc., which refer to chromosome numbers, should not be used to indicate DNA contents. Also ploidy data gained using FCM should be distinguished from that obtained via chromosome counting. If ploidy/aneuploidy is inferred from nuclear DNA content but is not confirmed by chromosome analysis, a prefix 'DNA' (i.e., 'DNA ploidy', 'DNA aneuploidy') should be used[36].

Is it really so easy?

Sometimes it is not, and the standard methods may fail with specific samples for a variety of reasons. The first trouble may be a difficulty to isolate sufficient intact nuclei for analysis. Such an inconvenience is often linked to the mechanical and chemical structure of the sample. For example, nuclei may be entrapped inside the cells or may stick to mucilaginous compounds[37]. Aggregation of fluorescent debris particles to the surfaces of isolated nuclei, i.e., so-called 'debris coatings'[34], is another phenomenon that results in decreased resolution of DNA content histograms[38]. A third, particularly acute problem concerns the interference of cytosolic compound(s) with the fluorescent staining of DNA, which usually results in decreased fluorescence intensity. Because the nuclei of the sample and the standard may be affected differentially[38], estimation of DNA content in absolute units may be compromised, as evidenced, for example, by poor

TABLE 2 | DNA reference standards recommended for the estimation of nuclear DNA amounts in absolute units.

Plant species and cultivar[a]	2C DNA content (pg DNA)[b]
Raphanus sativus L. 'Saxa'[32]	1.11
Solanum lycopersicum L. 'Stupické polní rané'[32]	1.96
Glycine max Merr. 'Polanka'[46]	2.50
Zea mays L. 'CE-777'[47]	5.43
Pisum sativum L. 'Ctirad'[33]	9.09
Secale cereale L. 'Daňkovské'[33]	16.19
Vicia faba L. 'Inovec'[32]	26.90
Allium cepa L. 'Alice'[33]	34.89

[a]Seeds may be obtained free of charge by contacting the corresponding author at dolezel@ueb.cas.cz. [b]2C DNA contents were determined using human leukocytes (2C = 7.0 pg) as a primary internal reference standard. *Note*: An alternative set of DNA reference standards was used by Johnston and co-workers[44].

reproducibility of results. Testing variants of protocols for nuclei isolation and different nuclei isolation buffers is advisable. The buffers can further be modified by manipulating the concentration of detergents and adding compounds counteracting the negative effects of cytosol (see the list of buffer compositions provided by Greilhuber and co-workers[34]). If the experiment does not require the analysis of nuclei from specific tissues, a useful strategy may be to identify less problematic tissues (e.g., embryo hypocotyl from mature dry seeds)[24].

Experimental design

This protocol describes the preparation of nuclear suspensions from fresh leaf tissues, *in vitro* cultured calli, protoplasts, mature seeds, herbarium vouchers and silica-dry materials, and flow cytometric estimation of DNA content in relative and absolute units (genome size). Particularities of FCM analysis of dehydrated plant tissues and mature seeds are described in detail elsewhere[39,40]. For the sake of clarity, protocols involving tissue and nuclei fixation are not covered here.

MATERIALS

REAGENTS

- Plant sample (fresh somatic plant tissue, *in vitro* cultured calli, living protoplasts, dry mature seeds, silica-dry material and herbarium vouchers) (see REAGENT SETUP)
- DNA reference standard (living plant somatic tissue); see **Table 2** for a list of recommended standards
- LB01 lysis buffer (see REAGENT SETUP)
- Galbraith's buffer (see REAGENT SETUP)
- Tris.$MgCl_2$ buffer (see REAGENT SETUP)
- Otto I solution (see REAGENT SETUP)
- Otto II solution (see REAGENT SETUP)
- PI stock solution (Sigma-Aldrich, cat. no. P4170; Molecular Probes, cat. no. P-3566) (see REAGENT SETUP) **! CAUTION** A mutagen and is moderately toxic. It may be harmful if inhaled, swallowed or absorbed through the skin. Use gloves when handling it. Be careful of particulate dust when weighing out the dye.
- RNase stock solution (Sigma-Aldrich, cat. no. R5000) (see REAGENT SETUP)
- DAPI stock solution (Sigma-Aldrich, cat. no. D9542; Molecular Probes, cat. no. D-1306) (see REAGENT SETUP) **! CAUTION** A possible carcinogen. It may be harmful if inhaled, swallowed or absorbed through the skin. It may also cause irritation. Use gloves when handling it. Be careful of particulate dust when weighing out the dye.
- Calibration particles: fluorescent beads (Molecular Probes, cat. no. A-7304—UV, Partec, cat. no. 05-4020—UV, Partec, cat. no. 05-4006—green) or stained trout red blood cells (Partec, cat. no. 05-7302—DAPI, Partec, cat. no. 05-7303—PI)
- Cleaning and decontamination solutions for flow systems (Becton Dickinson, cat. no. 340346; Partec, cat. nos. 04-4009 and 04-4010, weak solution of sodium hypochlorite, e.g., household bleach diluted 1:5 in distilled water)
- Optional: chemical compounds counteracting negative effects of cytosol such as reducing agents (e.g., β-mercaptoethanol) and phenolics-binding compounds (e.g., PVP-10, PVP-40)
- Tween 20 (Sigma-Aldrich, cat. no. P2287)
- NaOH
- Hydrochloric acid (HCl)

EQUIPMENT

- Plastic Petri dishes (5.5 cm diameter)
- Razor blades (double-edged) and razor blade holder (e.g., model GSM Mini-Glass Scraper; Allway Tools)
- Nylon mesh (42-μm pore size, squares 2 × 2 cm^2); uncut mesh (Uhelon mesh; Silk and Progress; Moravská Chrastová, CZ); Nytex mesh (Tetko Inc., Elmsford, NY). Alternatively, disposable filters (e.g., Partec, cat. nos. 04-0042-2316 or 04-0042-2317) may be used
- Polystyrene sample tubes suitable for the flow cytometer (e.g., BD Falcon, cat. no. 352008 for Becton Dickinson instruments and Sarstedt, cat. no. 55.484 for Partec instruments)
- Sample tube holder
- Ice container
- Air displacement pipettes and appropriate tips (1 ml, 200 μl)
- Flow cytometer with light source suitable for excitation of the DNA fluorochrome used in the study (e.g., diode-pumped solid-state laser emitting at 532 nm or argon-ion laser tuned to 488 or 514.5 nm for samples stained with PI, and a mercury arc lamp or laser tuned to 340–380 nm for samples stained with DAPI). While a one-parameter instrument is sufficient to measure DNA content, the ability to evaluate light scatter properties assists in assessing the possible negative effects of cytosolic compounds on sample quality[38]. In addition, doublets may be detected on a fluorescence/side scatter cytogram (see EQUIPMENT SETUP)
- Appropriate software for the evaluation of flow cytometric data
- Nitrile or latex gloves, safety glasses

REAGENT SETUP

Plant material If living plant tissues are used, they should be fresh, ideally sampled from intact, sufficiently watered plants, immediately before analysis. It may be important to choose appropriate developmental stage. Young, rapidly growing leaves usually give the best results; note that differentiated and senescent tissues may comprise a high proportion of endopolyploid nuclei.

Tissue samples can often be preserved for up to several days if kept at 4 °C in a Petri dish or plastic box, (partially) wrapped in a moistened paper tissue. The period for which a tissue can be stored differs in individual species and should be determined empirically. In some applications (e.g., DNA ploidy estimation), silica-dry materials, herbarium vouchers and mature seeds can be used. However, to avoid unspecified effects on chromatin and DNA staining due to drying or fixation, only fresh living tissues are recommended for high-quality estimation of DNA contents in absolute units.

In any case, materials potentially colonized by pests (fungi, insects and tissues with insect eggs laid on) harbor risk of biological contamination and accumulation of defence substances, and should therefore be avoided.

If protoplasts are used, they should be freshly isolated (to avoid regeneration of cell wall) and viable (greater than 90%). Nuclei cannot be isolated from 'collapsed' protoplasts, and it is recommended that protoplasts are purified (e.g., by gradient centrifugation) before use. Protoplast viability can be determined, for example, using fluorescein diacetate[41].

In vitro cultured calli are suitable if they are sufficiently compact and actively growing. Senescent and dying tissues (brown/black) should be avoided. Friable calli that completely disperse to single cells and small cell clusters after immersing in the nuclear isolation buffer are less appropriate since they are difficult to homogenize. Their suitability should be tested empirically.

LB01 buffer 15 mM Tris, 2 mM Na_2EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 0.1% (vol/vol) Triton X-100. Adjust to pH 7.5 with 1 M NaOH. Filter through a 0.22-μm filter. Add β-mercaptoethanol to 15 mM. Store at –20 °C in 10 ml aliquots. Do not refreeze after thawing. **! CAUTION** β-mercaptoethanol may be fatal if inhaled or absorbed through the skin, and is harmful if swallowed. High concentrations are destructive to the skin, eyes, mucous membranes and upper respiratory tract. Wear gloves and safety glasses and work in a chemical fume hood when handling concentrated solutions.

Galbraith's buffer 45 mM $MgCl_2$, 20 mM MOPS, 30 mM sodium citrate, 0.1% (vol/vol) Triton X-100. Adjust pH to 7.0 with 1 M NaOH, filter through a 0.22-μm filter and store at –20 °C in 10 ml aliquots. Do not refreeze after thawing.

Tris.$MgCl_2$ buffer 200 mM Tris, 4 mM $MgCl_2 \cdot 6H_2O$, 0.5% (vol/vol) Triton X-100. Adjust pH to 7.5 with 1 N HCl, filter through a 0.22-μm filter and store at 4 °C.

Otto I solution 0.1 M citric acid, 0.5% (vol/vol) Tween 20. Filter through a 0.22-μm filter and store at 4 °C. **▲ CRITICAL** It is essential to use cell culture–tested Tween 20 (Sigma-Aldrich, cat. no. P2287). Tween 20 for molecular biology (Sigma-Aldrich, cat. no. P9416) is not suitable for FCM and yields DNA content histograms with lower resolution.

Otto II solution 0.4 M $Na_2HPO_4 \cdot 12H_2O$. Filter through a 0.22-μm filter and store at room temperature (18–25 °C). Dissolving can be speeded up by heating. Note that a fluorochrome can be added before final volume adjustment. In that case, the solution should be stored in darkness.

DAPI stock solution 0.1 mg ml^{-1} DAPI. Filter through a 0.22-µm filter to remove small particles. Store at –20 °C in 1 ml aliquots. Do not refreeze after thawing.
PI stock solution 1 mg ml^{-1} PI. Filter through a 0.22-µm filter to remove small particles. Store at –20 °C in 1 ml aliquots. Do not refreeze after thawing.
RNase stock solution 1 mg ml^{-1} RNase. Heat to 90 °C for 15 min to inactivate DNases. Filter through a 0.22-µm filter. Store in 1 ml aliquots at –20 °C. Do not refreeze after thawing.

EQUIPMENT SETUP
Flow cytometer Check fluidics for proper function and make sure that the tubing is clean, free of air bubbles and that in-line filters are free of any blocking particles such as algae. *Note*: sodium azide (e.g., 0.02% wt/vol) may be added to the sheath fluid to prevent microbial growth in the reservoir. The optical filter set should be compatible with the spectral properties (i.e., excitation and emission spectra) of the DNA fluorochrome used. Align the instrument using appropriate calibration particles to obtain the lowest coefficient of variation of the fluorescent peaks (light scatter peaks if applicable) and lowest background, and check the instrument linearity (the 4C/2C peak ratio should be in the range of 1.98–2.02). The use of fluorescent beads is advisable as their quality is known and their analysis reflects the instrument behavior. While simple instruments do not require extensive adjustment of many parameters, more sophisticated cytometers may require setting appropriate threshold levels, gating windows and other parameters. In some instruments, fluorescence pulse parameters may be selected, such as pulse height and area. Although, in principle, pulse area should be selected, we recommend consulting manufacturer's instructions for appropriate setting (e.g., pulse height is the standard option in Partec instruments due to the large diameter of the excitation light beam).

PROCEDURE

Preparing suspensions of intact nuclei

1| Nuclear suspensions can be prepared either according to the one-step protocol (option A) or the two-step protocol (either option B or option C). While the one-step protocol presented here works with most plant species, for some of them the two-step protocol results in histograms with higher resolution of DNA peaks. If preparing nuclear suspensions from protoplasts, follow option D.

(A) Preparation of nuclear suspension using one-step protocol ● TIMING 5 min or more

(i) Place a small amount of plant tissue (typically 20 mg) in the center of a plastic Petri dish.
▲ CRITICAL STEP The amount of material used should be determined empirically with respect to the concentration of nuclei in the sample and the amount of debris background on the histogram of DNA content. For internal standardization, add leaf tissues of an appropriate reference standard. The ratio sample:standard tissue amount should be determined empirically, so that their G_1 peaks are of similar height on the histograms of DNA content.

(ii) Add 1 ml ice-cold nuclei isolation buffer (LB01, Galbraith's buffer, Tris.$MgCl_2$ buffer) to the Petri dish.
▲ CRITICAL STEP The choice of nuclear isolation buffer is empirical. It is recommended to test several buffers and select the best performing one for a given plant species and tissue.

(iii) Chop the tissue immediately in the buffer with a new razor blade or a sharp (disposable) scalpel.
▲ CRITICAL STEP It is important that the razor blade or scalpel is sharp, and the material is chopped into very fine slices and not just squeezed (use each edge of the razor blade only once). The extent of chopping should be determined empirically with respect to the concentration of nuclei in the sample and the amount of debris background on the histogram of DNA content. Dispersal and drying out the buffer during chopping should be avoided. Tilting the Petri dish so that the buffer occupies a small surface is helpful. Chopping the tissue in a walk-in cold room (with all components chilled to 4 °C) may be helpful in recalcitrant plant samples.

(iv) Mix the homogenate by pipetting up and down for several times (avoid air bubbles).

(v) Filter the homogenate through a 42-µm nylon mesh into a labeled sample tube. Note that due to losses of the solution during chopping and filtration, the volume of the filtrate is typically approximately 0.5 ml. If critical, sample loss can be reduced by presoaking the nylon mesh with nuclear isolation buffer shortly before filtration. Check visually that the filtrate is free of any particles that may cause instrument clogging.

(vi) Add stock solution of a DNA fluorochrome and shake gently. DAPI is typically used at 4 µg ml^{-1}; PI is typically used at 50 µg ml^{-1} simultaneously with RNase at 50 µg ml^{-1}.
▲ CRITICAL STEP As PI binds also to dsRNA, it needs to be used simultaneously with RNase.

(vii) Incubate the sample on ice before analysis (a few minutes to 1 h), with occasional shaking.
▲ CRITICAL STEP The optimal staining period for a given plant species and tissue should be determined experimentally.

(viii) Proceed to Step 2 to analyze nuclear DNA content.

(B) Preparation of nuclear suspension using a two-step protocol ● TIMING 15 min or more

(i) Follow Step 1A(i).

(ii) Add 1 ml ice-cold Otto I solution to the Petri dish.

(iii) Follow Step 1A(iii–v).

(iv) Pellet the nuclei (150 g/5 min).
▲ CRITICAL STEP As the isolated nuclei are stable in the Otto I solution, it is possible to prepare several samples in advance and centrifuge them simultaneously. The relative centrifugal force and duration of centrifugation may require adjustment for certain materials and should be verified empirically.

(v) Carefully remove the supernatant, leaving approximately 100 µl of the liquid above the pellet. Take care not to remove the pelleted nuclei.

(vi) Resuspend the nuclei by gentle shaking (flicking the bottom of the tube using a finger may help), and add 100 µl fresh ice-cold Otto I solution.
■ **PAUSE POINT** Store the suspension at room temperature or at 4 °C until the next step, shaking occasionally. The samples can be kept at this stage for up to several hours. This allows the preparation of many samples before the analysis and their transfer to a FCM laboratory if the samples cannot be prepared on site.

(vii) Add 1 ml Otto II solution to the nuclear suspension.
▲ **CRITICAL STEP** The addition of the Otto II solution should raise pH of the nuclear solution to approximately 7.3 (citric acid + phosphate buffer) and increase its salt concentration. To maintain these parameters within a reasonable range, the amount of the added Otto II should be approximately fourfold that of the nuclear solution. The sample should be analyzed within a few minutes of adding Otto II solution. Isolated nuclei may not be stable for prolonged periods after this step.

(viii) Add stock solution of a DNA fluorochrome and shake gently. DAPI is typically used at 4 µg ml^{-1}; PI is typically used at 50 µg ml^{-1} simultaneously with RNase at 50 µg ml^{-1}. Alternatively, the nuclear stains (and RNase) can be included in the Otto II solution.

(ix) Incubate the sample for a few minutes, with occasional shaking.
▲ **CRITICAL STEP** The optimal staining period for a given plant species and tissue should be determined experimentally. Short incubations often give the best results.

(x) Proceed to Step 2 to analyze nuclear DNA content.

(C) Preparation of nuclear suspension using a simplified two-step protocol ● TIMING 5 min or more

(i) Perform Step 1B(i–iii).

(ii) Add 2 ml of Otto II solution to the nuclear suspension.

(iii) Follow Step 1B(viii–x).

(D) Preparation of nuclear suspension from protoplasts ● TIMING 10 min or more

(i) Add freshly isolated and viable protoplasts to ice-cold nuclear LB01 isolation buffer to a concentration of 10^5–10^6 per ml.
▲ **CRITICAL STEP** In order to improve the release of nuclei from protoplasts, the concentration of detergent (Triton X-100) in the LB01 buffer should be increased to 0.5–2% (vol/vol). Suitable concentrations should be determined empirically. *Note*: Extremely high detergent concentrations may cause nuclear instability.

(ii) Incubate for 5–15 min on ice with occasional shaking of the tube.

(iii) Follow Step 1A(v–viii).

Analysis of nuclear DNA content

2| FCM measures the relative fluorescence of the stained nuclei. The basic protocol for sample analysis is presented below (option A). Options B and C describe ploidy estimation using external and internal standards, respectively, whereas option D describes the analysis of genome size in absolute units. For the sake of simplicity, only one-parameter analyses are considered.

(A) Measurement of relative nuclear DNA fluorescence intensity ● TIMING 5 min

(i) Introduce the suspension of stained nuclei into the flow cytometer and run it for a few seconds.
▲ **CRITICAL STEP** This period is required to stabilize sample rate and to equilibrate the concentration of dye bound to sample nuclei and to the line tubing.

(ii) Adjust the sample rate. Due to rather low concentration of nuclei in the sample, the sample rate usually does not exceed 20–50 nuclei/s.
▲ **CRITICAL STEP** The concentration of nuclei in the sample varies greatly depending on the quantity of source tissue and its type. It may be as low as 2×10^3 nuclei ml^{-1} in samples prepared from a small amount of silica-dried material, and reach 200×10^3 nuclei ml^{-1} in samples prepared from seeds and protoplasts. Excessive sample delivery rates may result in broadening DNA peaks and higher peak CVs. Note that some instruments only have a few pre-set sample delivery rates (e.g., slow, medium and fast). In that case, changing sample rate between samples is usually not necessary.

(iii) Position DNA peak(s) to the required position on the abscissa by adjusting the instrument gain settings (photomultiplier voltage, amplifier gain or both). A threshold for cutting-off undesirable low-channel signals coming from cell debris and/or autofluorescent compounds may be required.
▲ **CRITICAL STEP** The gain should be kept within the range recommended by the manufacturer to avoid signal anomalies due to saturation effects and other nonlinearities.

(iv) Measure 5,000 particles and save the data.

(v) Use appropriate software to assess the quality of analysis by estimating the proportion of debris background, which should be as low as possible, by checking peak symmetry and by evaluating the distribution of fluorescence intensities (width of

DNA peaks), expressed as coefficient variation (CV% = SD of the peak/mean channel position of the peak × 100). CVs below 3% are considered good, whereas measurements having CVs more than 5% are considered not acceptable. It should be noted that CVs below 5% cannot be achieved with some materials (e.g., samples rich in polyphenolics, with very small genome size, some herbarium vouchers). Consider that CVs can only be calculated for the measurements done in linear scale (CV values are meaningless for peaks on logarithmic scale).

▲ CRITICAL STEP This analysis is critical especially when a new material is measured as the data will be used to choose the optimal methodology.

(B) Ploidy analysis with external standard ● TIMING 5 min

(i) Run a sample prepared from a (reference) plant with known ploidy (chromosome number) following Step 2A(i and ii).

▲ CRITICAL STEP The reference plant must belong to the same species as the unknown sample(s). The plant may not necessarily be diploid. However, this needs to be considered when estimating DNA ploidy of unknown sample(s).

(ii) Using instrument gain (photomultiplier voltage, amplifier gain or both), position the peak of G_1 nuclei of a diploid reference plant on approximately one-fifth of the linear abscissa (e.g., channel 200 on a 1,024 scale).

▲ CRITICAL STEP Do not change instrument settings after this step. It is advisable to run new samples from the reference plant approximately each hour and re-establish its G_1 peak position.

(iii) Analyze unknown samples following Step 2A(i and ii) and 2A(iv and v).

▲ CRITICAL STEP Due to variation in sample preparation, staining and analysis, the peak position may not exactly reflect the ploidy. While in low polyploids a small shift in peak position usually does not compromise reliability of ploidy estimates, attention should be paid to the analysis of high polyploids (e.g., greater than 6*x*) because relative differences in nuclear DNA content between neighboring cytotypes decrease with increasing ploidy. Reliable estimation of ploidy levels may then require an internal standard (see option C). The use of internal standards is recommended whenever there are doubts about the results, and especially when novel and/or unexpected ploidy is found.

(iv) Determine the mean channel number of the G_1 sample peak.

(v) Calculate the DNA ploidy of the unknown sample as follows:

$$\text{Sample ploidy(integer)} = \text{Reference ploidy} \times \frac{\text{mean position of the } G_1 \text{ sample peak}}{\text{mean position of the } G_1 \text{ reference peak}}.$$

▲ CRITICAL STEP Note that the assay with external standard is not sufficiently precise for aneuploidy detection. This should be done with internal standardization (see option C).

■ PAUSE POINT Optionally, histogram evaluation (Step 2A(v)) can be postponed and performed off-line after data acquisition.

(C) Ploidy analysis with internal standard ● TIMING 5 min

(i) Prepare the sample by simultaneous chopping and staining tissues from a reference plant with known ploidy (chromosome number) and from the unknown plant sample as described earlier.

▲ CRITICAL STEP The reference plant should belong to the same species as the unknown sample(s). Because of this, nuclear DNA may be stained either by PI or DAPI, although the latter option may result in higher resolution of histograms of DNA content. The use of DAPI is also recommended if aneuploidy detection is attempted.

(ii) Perform sample analysis following Step 2A(i–v). Positioning the standard peak on an appropriate channel on the abscissa is usually done only once and the following samples are measured under the same instrument settings.

(iii) Determine the DNA ploidy of the unknown sample following Step 2B(iv and v).

▲ CRITICAL STEP Perfect overlap of peaks of the reference plant and the unknown sample indicates the same ploidy. Bifurcated or nonsymmetrical (skewed) peaks imply aneuploidy. However, aneuploidy may only be reliably identified using simultaneous analysis of a reference and aneuploid plant if the CVs of the DNA peaks are lower than half of the difference between the DNA contents of both plants[34,42]. If such a precision cannot be achieved, one solution is to use nuclei of a different plant species with an appropriate genome size as a reference point. In that case, aneuploidy identification requires replicate measurements[35]. Finally, it is always advisable to accompany unexpected flow cytometric results by conventional chromosome counts.

(D) Estimation of nuclear DNA content in absolute units (genome size) ● TIMING 5 min

(i) Select a suitable internal DNA reference standard by individually analyzing the DNA contents of the unknown sample and potential DNA reference standards.

▲ CRITICAL STEP Choose a reference standard whose genome size does not differ from that of the sample more than twofold. This reduces the risk of errors due to instrument nonlinearity. On the other hand, standards with too similar nuclear DNA content should not be used to avoid peak overlapping (this depends on the resolution of the DNA content histograms, the smallest reasonable difference being 15–20%). It is advisable to use one of the established plant DNA reference standards[43,44] (see **Table 2**).

BOX 1 | NEGATIVE EFFECT OF CYTOSOL ON THE ESTIMATION OF NUCLEAR DNA CONTENT

Some species contain cytosolic compounds (e.g., polyphenolics), which interfere with DNA staining and may distort FCM measurements. As the standard and sample species may differ in the amounts of these compounds as well as in sensitivity to their interference, it is advisable to assess potential negative effects by measuring the sample and standard first separately and then together[48]. Interference is detected if a shift in the peak position of the reference standard is observed after analyzing the mixed sample. If this is seen, other nuclei isolation buffers should be tested, as well as the use of compounds to ameliorate the negative effects. Moreover, the use of other parts of a plant and plants growing under different conditions (e.g., low light) should be considered (**Table 3**). A refined identification of negative cytosol effects involves the analysis of light scatter properties, which can be used to detect the so-called tannic acid effect[34,38].

(ii) Confirm the suitability of the reference standard by analyzing a sample prepared from both tissues simultaneously (see also **Box 1**).
▲ CRITICAL STEP This point provides the opportunity to detect the negative effects of cytosol on DNA staining.

(iii) Prepare the sample by simultaneously chopping and staining tissues from a DNA reference standard and unknown sample.
▲ CRITICAL STEP The protocol for sample preparation should be suitable for both DNA reference standard and unknown sample. Chopping should be done in a sandwich-like fashion rather than sequentially to make the effects of cytosolic compounds as equable as possible (J. Loureiro, personal communication). Because the standard and the sample may differ in DNA base content (AT/GC ratio), samples for the estimation of genome size should be stained by PI, the binding of which to DNA is not influenced by base composition.

(iv) Perform sample analysis following Step 2A(i–v). Positioning the standard peak on an appropriate channel on the abscissa is usually done only once, and the following samples are measured using the same instrument settings.

(v) The amount of nuclear DNA of the unknown sample is calculated as follows:

$$\text{Sample 2C value(DNA pg or Mbp)} = \text{Reference 2C value} \times \frac{\text{sample 2C mean peak position}}{\text{reference 2C mean peak position}}.$$

▲ CRITICAL STEP Take care of the C-levels of DNA peaks used for calculation. For example, in bryophyte gametophytes and in angiosperm haploids the first peak seen at the left of the histogram usually is in 1C.

(vi) Repeat the analysis on at least two other plants from the same accession. Alternatively, three replicates on the same plant may be performed if additional individuals are not available.
▲ CRITICAL STEP At least three different measurements should be done on different days to compensate for instrument drifts. The artifactual variation between particular FCM assays is usually below 2%, but may exceed this value in recalcitrant plant species.

(vii) Calculate the average 2C nuclear DNA content and perform statistical analysis as required. Mean DNA values should be accompanied by a measure of the spread of individual measurements (e.g., s.d., s.e.m. or maximum/minimum range) and the sample size.
▲ CRITICAL STEP Absolute differences between particular measurements (i.e., maximum/minimum value) should not pass the 2% threshold. In case of higher data variability, a possibility of plant material inhomogeneity should be checked (e.g., occurrence of chromosomally aberrant individuals, infected material). Outliers should be removed only upon a sound statistical justification, and their removal should be reported in a publication. Consequences of the removal for statistical significances must be carefully considered.

(viii) If needed, convert DNA mass in picograms to the numbers of base pairs or *vice versa* as follows[45]: 1 pg DNA = 0.978 × 10^9 bp.
▲ CRITICAL STEP Plant species are often polyploid and therefore a proper terminology for presenting genome size should be followed to avoid incorrect data interpretation[3] (see INTRODUCTION). Note that, in molecular biology, DNA quantity is often given in mega base (= DNA length) instead of mega base pair (= DNA mass), which can lead to a twofold error in converting DNA amounts to other units. Another source of potential twofold error may arise from the fact that molecular biologists commonly cite genome sizes in C-values, whereas cytometrists cite genome sizes in 2C-values.
? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**. **Box 1** discusses the possible solutions for situations where the cytosol has a negative effect on the estimation of nuclear DNA content.

TABLE 3 | Troubleshooting table.

Problem	Possible reasons	Solution
Precipitation of nuclear isolation buffer	Water used for buffer preparation was of poor quality	Use good-quality deionised water
Precipitation of the Otto II solution	The buffer was stored at a low temperature	Keep the buffer at room temperature
The sample turns brown/black	Negative effect of cytosol	Add reducing agents such as β-mercaptoethanol and/or add the tannin-binder PVP-10/PVP-40 (polyvinylpyrrolidone) to the buffer; reduce chopping intensity; test a different tissue; use a different nuclear isolation buffer
Small numbers of nuclei in the sample	The amount of sample tissue was not sufficient	Use larger amount of tissue
	Sample tissue was not chopped enough	Chop the tissue more extensively (note that the tissue must be chopped and not squeezed) and use a sharp razor blade/scalpel
	Nuclei adhere to mucilaginous compounds and do not pass through the nylon mesh	Use a different isolation buffer; increase detergent concentration; test a different tissue
	The tissue was not suitable for sample preparation	Test a different tissue
	Clogged flow chamber or line tubing/low pressure in the fluidics	Clean the flow chamber; exchange line tubing; clean in-line sheath fluid filter; change pressure in the system; check for leaks
No peaks on the histogram of relative fluorescence intensity	Improper setting of optical bench and control software	Use the filter combination suitable for a given fluorochrome; check instrument settings and performance using suitable calibration particles
	The peaks are outside the scale	Use log scale, adapt the region of interest on the scale, check system trigger and threshold settings
No peaks when using the two-step protocol	Wrong type of Tween 20	Use cell culture–tested Tween 20
	Sample stands too long after adding Otto II solution	Prepare a new sample
Large amount of debris background	Improper protocol for nuclei isolation	Use a different nuclear isolation buffer; test a different tissue
	Inappropriate length of sample staining	Change the staining period
	Razor blade or scalpel are not sharp enough	Use a new razor blade or scalpel
	The material was chopped too much	Reduce vigor of chopping
Broad DNA peaks (high coefficients of variation (CV))	Instrument is not aligned	Check instrument alignment using suitable particles, check system the flow chamber
	Air bubble in the flow chamber	Clean the flow chamber
	Obsolete arc lamp used for UV excitation	Replace the arc lamp
	Improper protocol for nuclei isolation	Use a different protocol/isolation buffer
	Recalcitrant tissue	Use a different tissue (e.g., with low metabolic activity or etiolated parts)
	Wilted plant tissue (low turgor)	Water the plant samples adequately before analyses
	Negative effect of cytosol	Add reducing agents such as β-mercaptoethanol and/or the tannin-binder PVP-10/PVP-40; reduce vigor of chopping; test a different tissue; use a different nuclear isolation buffer; prepare the samples in a walk-in cold room
	Absence of RNase when propidium iodide (PI) is used to stain DNA	Add RNase with PI (this is critical for the tissue with active protein synthesis, such as root tips)

TABLE 3 | Troubleshooting table (continued).

Problem	Possible reasons	Solution
	Incorrect concentration of DNA fluorochrome	Check the fluorochrome concentration
Weak fluorescence and broad DNA peaks when analyzing 4′,6-diamidino-2-phenylindole (DAPI)-stained samples after measuring samples stained with propidium iodide	Interference of remnants of propidium iodide in the sample line with DAPI	Remove the remnants of PI by washing the sample line with a weak solution of household bleach (i.e., approximately 1% sodium hypochlorite + detergent). Wash the bleach out thoroughly with deionised water before sample analysis
Prominent peak of G_2 nuclei	Analyzed vegetative tissues are old, dormant or too rapidly growing	Use young, but not too rapidly growing, tissue; repeat analyses in another season
Several peaks arranged in a nonendopolyploid fashion	Tissue contaminated by pests (e.g., insect eggs, rust, fungi)	Use tissue free of parasites and other contaminants

ANTICIPATED RESULTS

The assay should result in a histogram of fluorescence intensities, which correspond to nuclear DNA contents. Generally, the histograms contain only DNA peaks representing interphase nuclei. S-phase nuclei are rare in samples prepared from leaves, which is the most frequently used tissue. However, they are frequent in tissues with cycling cells such as root tip meristems. The interphase nuclei of cycling cells may be either in G_1 or in G_2 phases of the cell cycle, but the cycling cells may temporarily or irreversibly leave the cycle in G_1 or G_2 and enter quiescent states $G_{1/0}$ and $G_{2/0}$, respectively. As the nuclei of cycling and quiescent interphase cells have the same DNA amounts, and as it is not trivial to discriminate between quiescent and cycling nuclei, in the following we only refer to the G_1 and G_2 phases.

The number of DNA peaks on a histogram and their height depend primarily on the sample type. Analysis of freshly growing leaves typically results in histograms with a single prominent DNA peak representing nuclei in G_1 phase of the cell cycle and a minor, sometimes undetectable, peak representing G_2-phase nuclei (**Fig. 1a**). In certain instances, the G_1 peak may be undetectable and only the G_2 peak may be seen. The analysis of older and dormant leaves may reveal a large peak of nuclei arrested in G_2 phase (**Fig. 1b**). However, similar results may also be obtained in a mixoploid plant consisting of diploid and tetraploid cells (**Fig. 1c**). Many plant species exhibit endopolyploidy, and the presence of endopolyploid nuclei is demonstrated by additional peaks with 8C, 16C, 32C and even higher DNA levels (**Fig. 1d**). While the DNA content analysis is typically done using linear amplification (**Fig. 1a–c**), analysis of higher levels of endopolyploidy may be facilitated by using a logarithmic scale for the abscissa (**Fig. 1d**).

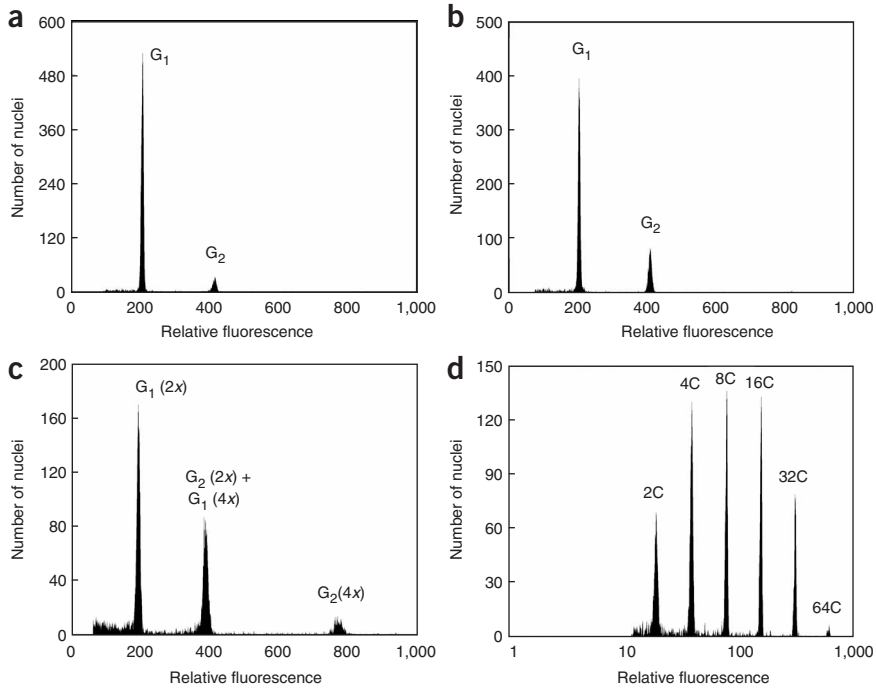

Figure 1 | Histograms of relative fluorescence intensities (relative nuclear DNA contents) obtained after the analysis of isolated plant nuclei. All samples were prepared according to simplified two-step method (Step 1C), stained with 4′,6-diamidino-2-phenylindole and measured using a Partec PAII flow cytometer equipped with mercury arc lamp as the excitation light source. (**a**) Analysis of nuclei isolated from a leaf of *Pisum sativum* yielded a histogram with a single dominant peak of G_1-phase nuclei. (**b**) The analysis of an older leaf of *Zea mays* revealed a higher proportion of nuclei within G_2 phase. (**c**) A similar histogram is obtained after analyzing a mixoploid plant of *Kochia scoparia* ($2n = 2x + 4x$) with diploid and tetraploid cells. (**d**) The analysis of endopolyploidy in *Senecio rowleyanus* was facilitated by the use of a logarithmic abscissa, which permitted visualization of higher numbers of endopolyploidy levels.

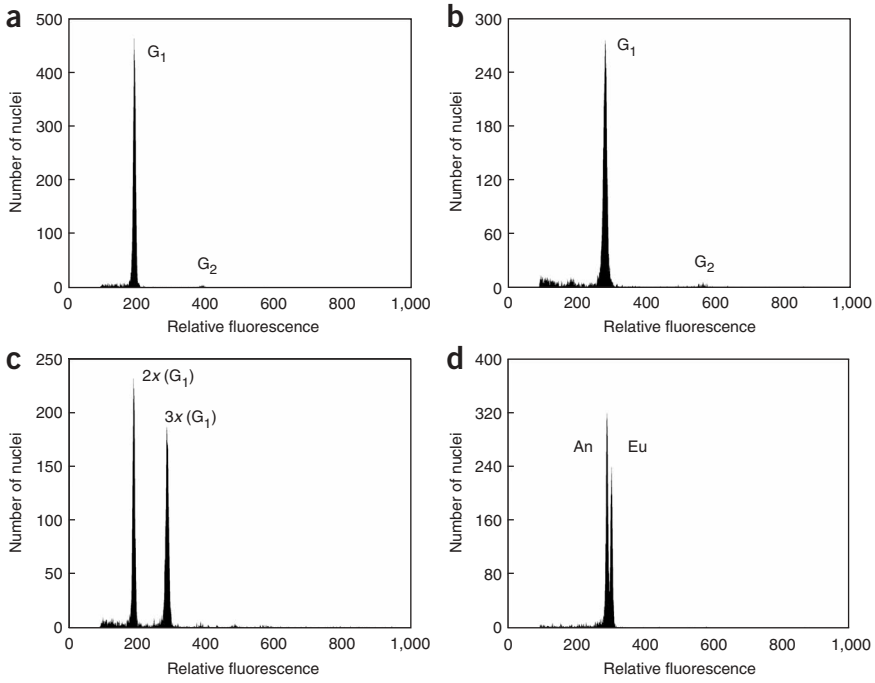

Figure 2 | Flow cytometric ploidy analysis. Ploidy was analyzed in *Hieracium echioides* using external (panels **a** and **b**) and internal (panels **c** and **d**) standards. (**a**) A diploid plant ($2n = 2x = 18$) was used as an external reference standard and its G_1 peak was positioned at channel 200. (**b**) The G_1 peak of the unknown sample appeared on channel 296, and its ploidy was thus estimated as $3x$. (**c**) This conclusion was confirmed after simultaneous analysis of the reference diploid plant and the triploid sample, when the ratio of G_1 peak positions was 1.506. (**d**) Aneuploidy status of a *Hieracium* hybrid plant (An) was suggested after its simultaneous analysis with an euploid hybrid of the same origin ($2n = 45$; Eu). Subsequent karyological analyses of the unknown sample revealed 43 chromosomes in the somatic tissues. The samples in panels (**a**–**c**) were prepared according to Step 1A and using LB01 buffer; sample (**d**) was prepared according to the standard two-step method (Step 1B). Samples were stained with 4′,6-diamidino-2-phenylindole (DAPI) and measured on a Partec PAII flow cytometer equipped with a mercury arc lamp as the excitation light source.

Ploidy screening is one of the most frequent applications of DNA FCM. The assay may be performed either with external (**Fig. 2a** and **b**) or internal standards (**Fig. 2c** and **d**). While the former is suitable for large-scale screening, the latter is more precise since it eliminates most of the sources of variation. External standardization involves the analysis of a plant with known ploidy (**Fig. 2a**). The analysis of an unknown conspecific plant with the same instrument settings results in a histogram of DNA content whose relative G_1 peak position indicates the plant ploidy (**Fig. 2b**). Simultaneous analysis of a reference and unknown plant (internal standardization) of different ploidy results in a histogram having two G_1 peaks. The ratio of the G_1 peak positions reflects the ratio of ploidy levels (**Fig. 2c**). Provided the resolution of a DNA content histogram is sufficiently high, it is possible to discriminate the peaks representing a diploid standard and an aneuploid plant (**Fig. 2d**).

Estimation of genome size in absolute units requires internal standardization. The reference standard may have a genome size reasonably larger (**Fig. 3a**) or smaller (**Fig. 3b**) than the unknown sample. In both cases, the histograms of DNA content should comprise two large G_1 peaks representing nuclei of the reference and the sample. The ratio of their positions determines the genome size of the unknown sample.

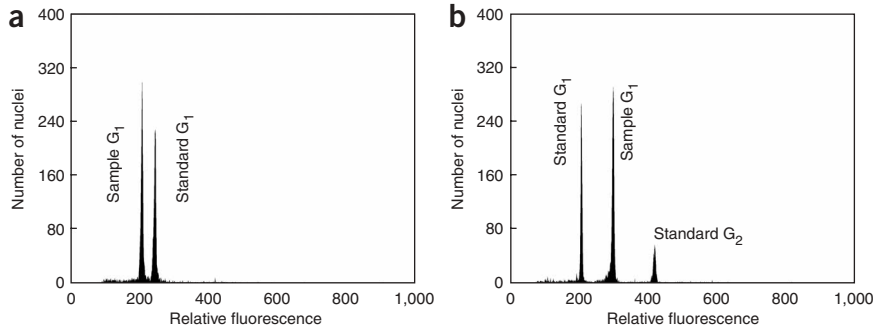

Figure 3 | Estimation of nuclear DNA content in absolute units. Examples are given for the analysis with internal reference standards having larger (**a**) and smaller (**b**) genome sizes as compared with the unknown sample. (**a**) The genome size of hexaploid *Curcuma angustifolia* was 0.847-fold that of the standard (*Glycine max* 'Polanka'; 2C = 2.50 pg DNA), and its 2C-value was estimated as 2.12 pg DNA. (**b**) The genome size of tetraploid *Hieracium aurantiacum* was 1.440-fold of that of the standard (*Zea mays* 'CE-777'; 2C = 5.43 pg DNA), and its 2C-value was determined as 7.82 pg DNA. The samples were prepared according to the two-step method (Step 1B), stained with propidium iodide (+RNase) and measured on a Partec CyFlow cytometer equipped with a Cobolt diode-pumped solid-state laser emitting at 532 nm.

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1. Doležel, J., Greilhuber, J. & Suda, J. Flow cytometry with plants: an overview. in *Flow Cytometry with Plant Cells* (eds. Doležel, J., Greilhuber, J. & Suda, J.) 41–65 (Wiley-VCH, Weinheim, Germany, 2007).
2. Loureiro, J., Suda, J., Doležel, J. & Santos, C. FLOWER: a plant DNA flow cytometry database. in *Flow Cytometry with Plant Cells* (eds. Doležel, J., Greilhuber, J. & Suda, J.) 423–438 (Wiley-VCH, Weinheim, Germany, 2007).

3. Greilhuber, J., Doležel, J., Lysák, M.A. & Bennett, M.D. The origin, evolution and proposed stabilization of the terms 'genome size' and 'C-value' to describe nuclear DNA contents. *Ann. Bot. (Lond.)* **95**, 255–260 (2005).
4. Greilhuber, J. *et al.* Smallest angiosperm genomes found in Lentibulariaceae, with chromosomes of bacterial size. *Plant Biol. (Stuttg).* **8**, 770–777 (2006).
5. Leitch, I.J., Soltis, D.E., Soltis, P.S. & Bennett, M.D. Evolution of DNA amounts across land plants (Embryophyta). *Ann. Bot. (Lond.)* **95**, 207–217 (2005).
6. Gregory, T.R. Coincidence, co-evolution or causation? DNA content, cell size and the C-value enigma. *Biol. Rev. Camb. Philos. Soc.* **76**, 65–101 (2001).
7. Bennett, M.D. Nuclear DNA content and minimum generation time in herbaceous plants. *Proc. R. Soc. Lond. B. Biol. Sci.* **181**, 109–135 (1972).
8. Leitch, I.J. & Bennett, M.D. Genome size and its uses: the impact of flow cytometry. in *Flow Cytometry with Plant Cells* (eds. Doležel, J., Greilhuber, J. & Suda, J.) 153–176 (Wiley-VCH, Weinheim, Germany, 2007).
9. Kron, P., Suda, J. & Husband, B.C. Applications of flow cytometry to evolutionary and population biology. *Annu. Rev. Ecol. Evol. Syst.* **38**, 847–876 (2007).
10. Rabinowicz, P.D. & Bennetzen, J.L. The maize genome as a model for efficient sequence analysis of large plant genomes. *Curr. Opin. Plant Biol.* **9**, 149–156 (2006).
11. Suda, J., Kron, P., Husband, B.C. & Trávníček, P. Flow cytometry and ploidy: applications in plant systematics, ecology and evolutionary biology. in *Flow Cytometry with Plant Cells* (eds. Doležel, J., Greilhuber, J. & Suda, J.) 103–130 (Wiley-VCH, Weinheim, Germany, 2007).
12. Matzk, F. Reproduction mode screening. in *Flow Cytometry with Plant Cells* (eds. Doležel, J., Greilhuber, J. & Suda, J.) 131–152 (Wiley-VCH, Weinheim, Germany, 2007).
13. Barow, M. & Meister, A. Endopolyploidy in seed plants is differently correlated to systematics, organ, life strategy and genome size. *Plant Cell Environ.* **26**, 571–584 (2003).
14. Barow, M. & Jovtchev, G. Endopolyploidy in plants and its analysis by flow cytometry. in *Flow Cytometry with Plant Cells* (eds. Doležel, J., Greilhuber, J. & Suda, J.) 349–372 (Wiley-VCH, Weinheim, Germany, 2007).
15. Barow, M. & Meister, A. Lack of correlation between AT frequency and genome size in higher plants and the effect of nonrandomness of base sequences on dye binding. *Cytometry* **47**, 1–7 (2002).
16. Meister, A. & Barow, M. DNA base composition of plant genomes. in *Flow Cytometry with Plant Cells* (eds. Doležel, J., Greilhuber, J. & Suda, J.) 177–215 (Wiley-VCH, Weinheim, Germany, 2007).
17. Pfosser, M., Magyar, Z. & Bögre, L. Cell cycle analysis in plants. in *Flow Cytometry with Plant Cells* (eds. Doležel, J., Greilhuber, J. & Suda, J.) 323–347 (Wiley-VCH, Weinheim, Germany, 2007).
18. Sgorbati, S., Sparvoli, E., Levi, M., Chiatante, D. & Giordano, P. Bivariate cytofluorometric analysis of DNA and nuclear protein content. *Protoplasma* **144**, 180–184 (1988).
19. Zhang, C.Q., Gong, F.C., Lambert, G.M. & Galbraith, D.W. Cell type-specific characterization of nuclear DNA contents within complex tissues and organs. *Plant Methods* **1**, 7 (2005).
20. Darzynkiewicz, Z., Bedner, E., Li, X., Gorzyca, W. & Melamed, M.R. Laser-scanning cytometry: a new instrumentation with many applications. *Exp. Cell Res.* **249**, 1–12 (1999).
21. Ulrich, I. & Ulrich, W. High-resolution flow cytometry of nuclear DNA in higher plants. *Protoplasma* **165**, 212–215 (1991).
22. Galbraith, D.W. *et al.* Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* **220**, 1049–1051 (1983).
23. Suda, J. & Trávníček, P. Reliable DNA ploidy determination in dehydrated tissues of vascular plants by DAPI flow cytometry—new prospects for plant research. *Cytometry A* **69**, 273–280 (2006).
24. Śliwińska, E., Zielińska, E. & Jedrzejczik, I. Are seeds suitable for flow cytometric estimation of plant genome size? *Cytometry A* **64**, 72–79 (2005).
25. Nsabimana, A. & van Staden, J. Ploidy investigation of bananas (*Musa* spp.) from the National Banana Germplasm Collection at Rubona-Rwanda by flow cytometry. *S. Afr. J. Bot.* **72**, 302–305 (2006).
26. Heller, F.O. DNS-Bestimmung an Keimwurzeln von *Vicia faba* L. mit Hilfe der Impulscytophotometrie. *Ber. Deutsch. Bot. Ges.* **86**, 437–441 (1973).
27. Sgorbati, S., Levi, M., Sparvoli, E., Trezzi, F. & Lucchini, G. Cytometry and flow cytometry of 4′,6-diamidino-2-phenylindole (DAPI)-stained suspensions of nuclei released from fresh and fixed tissues of plants. *Physiol. Plantarum* **68**, 471–476 (1986).
28. Levi, M., Sparvoli, E., Sgorbati, S. & Chiatante, D. Rapid immunofluorescent identification of cells in the S phase in pea root meristems: an alternative to autoradiography. *Physiol. Plantarum* **71**, 68–72 (1987).
29. Jarret, R.L. *et al.* DNA contents in *Paspalum* spp. determined by flow-cytometry. *Genet. Resour. Crop Evol.* **42**, 237–242 (1995).
30. Esteban, J.M. *et al.* Effects of various fixatives and fixation conditions on DNA ploidy analysis—a need for strict internal DNA standards. *Am. J. Clin. Pathol.* **95**, 460–466 (1991).
31. Shapiro, H. *Practical Flow Cytometry* 4th edn. (Wiley-Liss, New York, 2003).
32. Doležel, J., Sgorbati, S. & Lucretti, S. Comparison of three DNA fluorochromes for flow-cytometric estimation of nuclear DNA content in plants. *Physiol. Plantarum* **85**, 625–631 (1992).
33. Doležel, J. *et al.* Plant genome size estimation by flow cytometry: inter-laboratory comparison. *Ann. Bot. (Lond.)* **82** (Suppl A), 17–26 (1998).
34. Greilhuber, J., Temsch, E.M. & Loureiro, J.C.M. Nuclear DNA content measurement. in *Flow Cytometry with Plant Cells* (eds. Doležel, J., Greilhuber, J., Suda, J.) 67–101 (Wiley-VCH, Weinheim, Germany, 2007).
35. Roux, N., Toloza, A., Radecki, Z., Zapata-Arias, F.J. & Doležel, J. Rapid detection of aneuploidy in *Musa* using flow cytometry. *Plant Cell Rep.* **21**, 483–490 (2003).
36. Suda, J., Krahulcová, A., Trávníček, P. & Krahulec, F. Ploidy level versus DNA ploidy level: an appeal for consistent terminology. *Taxon* **55**, 447–450 (2006).
37. Loureiro, J., Rodriguez, E., Doležel, J. & Santos, C. Comparison of four nuclear isolation buffers for plant DNA flow cytometry. *Ann. Bot. (Lond.)* **98**, 679–689 (2006).
38. Loureiro, J., Rodriguez, E., Doležel, J. & Santos, C. Flow cytometric and microscopic analysis of the effect of tannic acid on plant nuclei and estimation of DNA content. *Ann. Bot. (Lond.)* **98**, 515–527 (2006).
39. Suda, J. & Trávníček, P. Estimation of relative nuclear DNA content in dehydrated plant tissues by flow cytometry. in *Current Protocols in Cytometry* (eds. Robinson, J.P. *et al.*) 7.30.1–7.30.14 (John Wiley & Sons, New York, 2006).
40. Śliwińska, E. Nuclear DNA content analysis of plant seeds by flow cytometry. in *Current Protocols in Cytometry* (ed. Robinson, J.P. *et al.*) 7.29.1–7.29.13 (John Wiley & Sons, New York, 2006).
41. Galbraith, D.W. Protoplast analysis using flow cytometry and sorting. in *Flow Cytometry with Plant Cells* (eds. Doležel, J., Greilhuber, J. & Suda, J.) 231–250 (Wiley-VCH, Weinheim, Germany, 2007).
42. Doležel, J. & Göhde, W. Sex determination in dioecious plants *Melandrium album* and *M. rubrum* using high-resolution flow cytometry. *Cytometry* **19**, 103–106 (1995).
43. Doležel, J. & Bartoš, J. Plant DNA flow cytometry and estimation of nuclear genome size. *Ann. Bot. (Lond.)* **95**, 99–110 (2005).
44. Johnston, J.S., Bennett, M.D., Rayburn, A.L., Galbraith, D.W. & Price, H.J. Reference standards for determination of DNA content of plant nuclei. *Am. J. Bot.* **86**, 609 (1999).
45. Doležel, J., Bartoš, J., Voglmayr, H. & Greilhuber, J. Nuclear DNA content and genome size of trout and human. *Cytometry A* **51**, 127–128 (2003).
46. Doležel, J., Doleželová, M. & Novák, F.J. Flow cytometric estimation of nuclear DNA amount in diploid bananas (*Musa acuminata* and *M. balbisiana*). *Biol. Plantarum* **36**, 351–357 (1994).
47. Lysák, M.A. & Doležel, J. Estimation of nuclear DNA content in *Sesleria* (Poaceae). *Caryologia* **52**, 123–132 (1998).
48. Price, H.J., Hodnett, G. & Johnston, J.S. Sunflower (*Helianthus annuus*) leaves contain compounds that reduce nuclear propidium iodide fluorescence. *Ann. Bot. (Lond.)* **86**, 929–934 (2000).