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Flow cytometry as tool in plant sciences, with emphasis on genome size and ploidy level assessment

Mickaël Bourge[1*], Spencer C. Brown[1], Sonja Siljak-Yakovlev[2]

[1] *Institute of Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Saclay, 91198, Gif-sur-Yvette, France*
[2] *Ecologie Systématique Evolution, Univ. Paris-Sud, CNRS, AgroParisTech, Université Paris-Saclay, 91400, Orsay, France*

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***Correspondence**
E-mail: Mickael.BOURGE@i2bc.paris-saclay.fr

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Abstract

Flow cytometry has become the method of choice to measure the DNA content (genome size) in plants. Ease of sample preparation, fast acquisition, and accurate measurements have made the method popular in the domains of plant cell biology, systematics, evolution, genetics and biotechnology. Although the cell wall is a problem when isolating plant cells, cytometry remains a powerful tool in plant sciences. Based on our 30-years' experience in this field, this review will focus at first on genome size measurement using simply isolated nuclei: the good practice for acquisition, nuclei isolation, appropriate buffers, kind of tissues to use. The second part will briefly review what kind of measurements it is possible to make in plant cytometry, and for what purpose: base composition, ploidy level, cell cycle, endoreplication, seed screening, and nuclei/chromosomes sorting. We will address troubleshooting. The commonly-used mathematical tools will be discussed.

Keywords

C-value, flow cytometry, genome size, gif nuclear isolation buffer, ploidy level, fluorescence

Introduction

The nuclear DNA amount (2C-value) or genome size is measured in picograms (1 x 10^{-9} g) or megabase pairs (Mbp, where 1pg=~978 Mbp according to Doležel et al., 2003). Genome size is a highly relevant biological character of living organisms and it is frequently correlated with many biotic and abiotic characters (Bennettet & Leitch, 2005; Pustahija et al., 2013). The term C-value was proposed by Swift (1950) to define the DNA content of the unreplicated gametic chromosome set of an organism. This value has been considered characteristic and invariable (the C for constant) for each species. However, since then, intraspecific genome size variation has often been detected (Vekemans et al., 1996; Gregory, 2005; Leitch et al., 2013, and references therein). Several terms have been formulated to represent different concepts in this field (Greilhuber et al., 2005).

Information on C-value can be fruitfully utilized in numerous branches of plant science. It is also a useful trait in ecology and phytogeography (Grime & Mowforth, 1982; Vekemans et al., 1996; Pustahija et al., 2013), systematics and evolution (Cerbah et al., 1999; Bogunic et al., 2011; Niketic et al., 2013; Lepers-Andrzejewski et al., 2011; Hajrudinovic et al., 2015), in biotechnology (Fyad-Lameche et al, 2016), agronomical (Srisuwan et al., 2018), horticultural (Cerbah et al., 2001; Hajrudinovic et al., 2015) and forestry sciences (Zoldos et al., 1998; Bou Dagher-Karatt et al., 2001; Siljak-Yakovlev et al., 2002; Bogunic et al., 2003; Siljak-Yakovlev et al., 2014), and also in evaluation of biodiversity (Bennett et al., 2000; Siljak-Yakovlev et al., 2010; Bou Dagher-Karatt et al., 2013; Siljak-Yakovlev et al., 2017).

Many arguments make flow cytometry the method of choice in wide applications of plant genome size estimation (Marie & Brown, 1993; Kamaté et al., 2001; Doležel et al., 2007; Siljak-Yakovlev et al., 2008; Siljak-Yakovlev et al., 2010; Bareka et al., 2012; Pellicer & Leitch, 2014). This method is rapid, ease of sample preparation and accurate for detection of small differences in DNA content (Benmiloud-Mahieddine et al., 2011; Karrat-Souissi et al., 2013; Pellicer & Leitch, 2014). Cytometry also facilitates rapid screening of genome size in several populations of the same species and on several individuals per population, favoring discovery of any polyploidy or hybridization events (Siljak-Yakovlev et al., 2008; Hajrudinovic et al., 2015).

However, despite its importance, the genome size has been estimated for only 3.1% of all angiosperms and 41% of gymnosperms (Pellicer et al., 2018). Evidently, there is still a wide demand for more coverage of DNA estimates for higher plants, especially for angiosperms.

The principle databases concerning plant genome size are presented in the Kew plant DNA C-values database (http://data.kew.org/cvalues), FLOWer, a plant DNA flow cytometry database (https://botany.natur.cuni.cz/flower/index.php), and GSAD, genome size in the Asteraceae database (http://www.etnobiofic.cat/gsad_v2/).

Materials and methods

1. Good practices for genome size measurement

It is easy to measure genome size of plant cells in cytometry, like others eukaryotic cells. Numerous measurements may be made in one day, without the

Table 1. Plants convenient for regular standard leaf tissue

Common name[a]	Specific name	2C DNA (pg) [b]	Base composition (GC %)
Arabidopsis	*Arabidopsis thaliana* L. Heynh ecotype Columbia or Bensheim	0.33	40.3
sage	*Salvia brachyodon* Vandas	0.95	38.5
barrel medic	*Medicago truncatula* Gaertn. cv R108-1	0.98	38.1
rice	*Oryza sativa* L. "IR-36"	1.00	-
rice	*Oryza sativa ssp. japonica 'Nipponbare'*	0.90	-
tomato	*Solanum lycopersicum* L. cv Montfavet 63-5	1.99	40.0
petunia	*Petunia hybrida* (Hort.) PxPc6	2.85	41.0
lucerne A2	*Medicago sativa* L. subsp *x varia* (Martyn) Arcangeli cv Rambler A2	3.47	38.7
sweet pea	*Pisum sativum* L. cv Express long	8.37	40.5
barley	*Hordeum vulgare* L.cv Sultan	9.81	-
artemisia	*Artemisia arborescens* L. (origin Crête)	11.43	-
wheat	*Triticum aestivum* L. cv Chinese Spring or Triple Dirk	30.9	43.7

[a] These are diploid except tetraploid lucerne and hexaploid wheat.

[b] All values come from Marie & Brown (1993) except *Artemisia arborescens* from Garcia et al. (2006), barley (cv Sultan) from Garnatje et al. (2004). This is a value for barley established in common between Barcelona and CNRS Gif-sur-Yvette. As noted by other authors, the long-standing value 2C=11.10 pg assigned by Bennett & Smith (1976) was probably an over-estimation: by 12%, we believe. Our barley seed originates from Kew (Jakob et al., 2004), *Salvia brachyodon* (origin Mt St Ilija, Croatia) from Siljak-Yakovlev et al. (2010), and *Oryza sativa* "IR-36" from Bennett & Smith (1991), and *Oryza sativa* ssp. *japonica* 'Nipponbare' (Uozu et al., 1997). Seeds are available from MB or SSY.

Note that 1 pg DNA = ~ 978 Mbp (from Doležel et al. 2003).

Table 2. DNA dyes and their specificity for cytometry. MW, molecular weight; Ex, peak of excitation; Em, peak of emission. These are all toxic for human use.

Dye	MW	Ex (nm)	Em (nm)	Specificity
Hoechst 33342	615.99	350	461	lipophilic; bound to A-T
Hoechst 33258	623.99	352	461	bound to A-T
DAPI	320.25	358	461	bound to A-T
Vybrant® DyeCycle™ Violet		369	437	lipophilic; stoechiometric
Hoechst 33580	590.96	382	440	bound to A-T
Chromomycin A_3	1183	440	555	bound to G-C
Mithramycin	1085	440	575	bound to G-C
YOYO-1		491	509	needs RNAse; stoechiometric
YO-PRO-1		491	509	needs RNAse; stoechiometric
SYTOX® Green	600	504	523	needs RNAse; stoechiometric
Vybrant® DyeCycle™ Green		506	534	lipophilic; stoechiometric
TOTO-1		514	533	needs RNAse; stoechiometric
TO-PRO-1		515	531	needs RNAse; stoechiometric
Ethidium bromide	394.32	518	605	needs RNAse; stoechiometric
Vybrant® DyeCycle™ Orange		519	563	lipophilic; stoechiometric
Propidium iodide	668.4	535	617	needs RNAse; stoechiometric
7AAD	1270.5	546	647	bound to G-C
DRAQ5	412.5	622	641	lipophilic; stoechiometric
TO-PRO-3	671.42	642	661	needs RNAse; stoechiometric
Rhodamine 700 (LD700)	538	659	669	bound to G-C
Oxazine 750	469.92	690	699	needs RNAse; stoechiometric
Rhodamine 800	431.96	700	715	bound to G-C

difficulties of cytogenetics which, nevertheless, remain essential to validate the cytometric estimation.

To make such measurements, it is necessary to have a panel of known standards, with mention of variety. Intra-species differences are regular, especially in cultivated plants. Therefore, it is essential to have some standard species available, as shown in table 1. It is better to have a standard with a genome size bigger than that of the specimen to measure: an interpolation will be preferable to an extrapolation.

The fluorescence of certain DNA dyes is dependent on the base composition as well as the DNA content (Table 2): these fluorochromes give approximate size. Intercalating dyes with simple stoichiometry (ethidium bromide, propidium iodide - PI) are necessary to measure absolute DNA content because they are insensitive to differences in bases composition. For example, comparing wheat and petunia, their genome sizes are 10.8-fold different measured with propidium iodide. The same analysis made with bisbenzimide Hoechst, which is fluorescent on five sequential AT, gives only an 8.6-fold difference, *i.e.* an error of 21%, because the wheat genome is less rich in AT bases than petunia's (Table 1).

For genome size measurement from fresh plant parts (mostly leaves but also stems, roots, etc.), both nuclei from sample and standard are simultaneously extracted, where the standard genome size is precisely known (Table 1), and DNA is labelled with stoichiometric dye (Figure 1). Quantity of dye to use is dependent on genome size to assure saturation [10 µg/ml of PI for *Arabidopsis thaliana* (0.33 pg), 50 µg/ml for tomato (1.99 pg) and petunia (2.85 pg), 100 µg/ml for wheat (30.9 pg)]. It is important for sample and standard tissues to be chopped together at the same time. The toxic dye is added after chopping. The samples should comprise several individuals, measured separately. To check the reproducibility of values, two distinct measurements should be performed for each individual.

Choice of nuclear isolation buffer

For nuclei preparation, choice of the buffer is essential. Galbraith buffer is common, containing 45 mM $MgCl_2$, 30 mM Sodium-Citrate and 20 mM MOPS acid pH 7.0 (Galbraith et al., 1983).

The buffer is often modified by adding 0.5 % Triton X-100 (to open up chloroplasts and mitochondria), even 1% to 2% for tissues comprising many terpenes. The use of 1% (m/v) polyvinyl-pyrrolidone (PVP) 10.000 complexes fluorescent polyphenols and prevents their polymerization and browning. A reducing agent stabilizes samples: commonly β-mercaptoethanol, but this is toxic and unpleasant, so

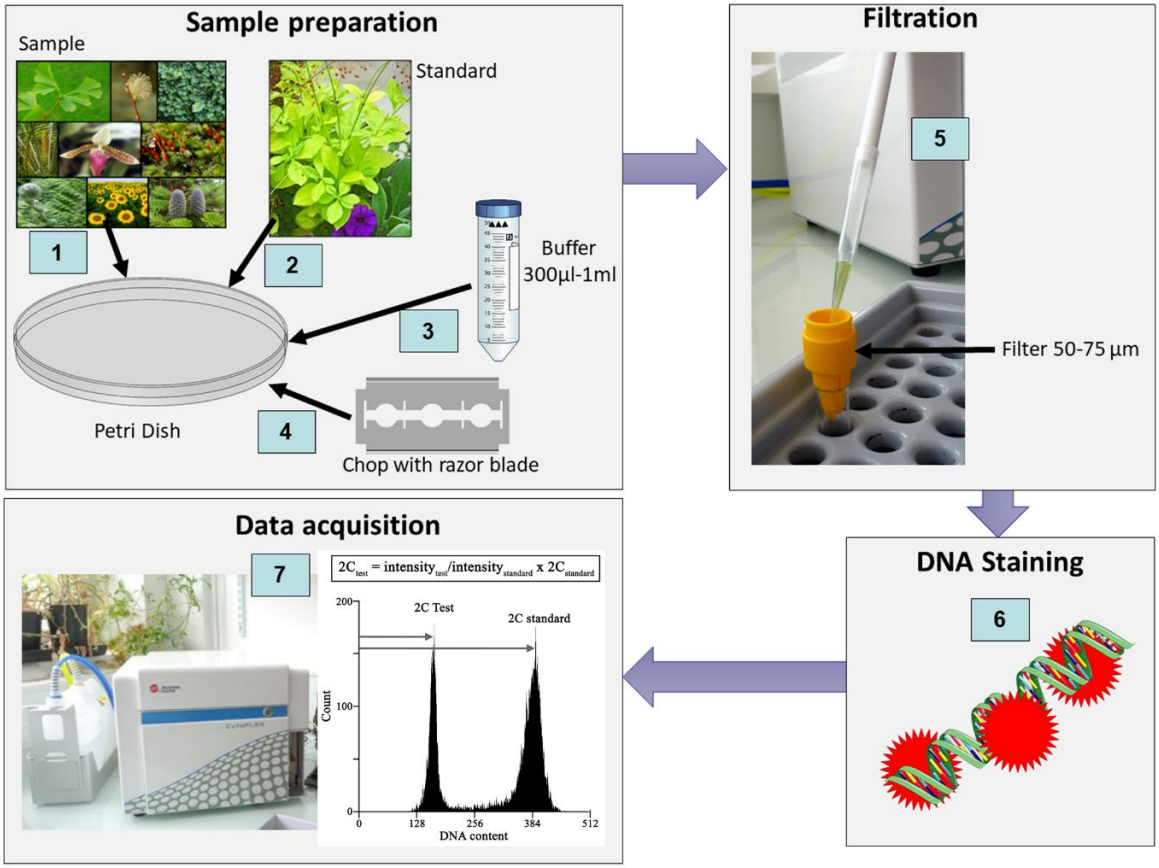

Figure 1. Tissues (leaves most of the time, approx. 30 mg) of both internal standard and target species are simultaneously chopped using a razor blade in a plastic Petri dish with 1 ml of buffer. The nuclei suspension is filtered through (30 or 50 µm nylon) mesh. The nuclei are stained with 10 to 100 µg/ml propidium iodide (PI), or other specific DNA intercalating fluorochrome dye, and kept 5 min at 4˚C. Results are shown as histograms with fluorescence intensity (DNA content) on x-axis and number of events (count) on y-axis. At least 2 peaks should be analyzed for their position on the X-axis, one represents the unknown value, the other represents the standard which allows 2C DNA calculation.

preferably fresh sodium metabisulfite is used.

In some cases, 20 mM MOPS is not enough for pH buffering (young tomato fruit for example), and acidic pH can alter DNA histogram, especially the coefficient of variation (Figure 2, Table 3). The Gif Nuclear Buffer (GNB), contains 45 mM $MgCl_2$, 30 mM Sodium-Citrate and 60 mM MOPS pH 7.0, 1% PVP 10.000, 0.1% Triton X-100 and 10 mM sodium metabisulfite ($S_2O_5Na_2$). This GNB buffer can be raised to 0.5% Triton X-100 or more, as necessary. This buffer can be stored, but the metabisulfite is added daily.

Isolated nuclei can be conserved many hours at 4°C by adding 1% to 2% formaldehyde (keep the formaldehyde stock at room temperature). Other buffers are detailed in Coba de la Peña & Brown (2001). It is also possible to process frozen tissue (by liquid nitrogen, then stored at -20°C), dry leaves and seeds, or material fixed with citric acid. Similar procedures can be applied in microbiology, chopping mycelium, for example, with internal standards.

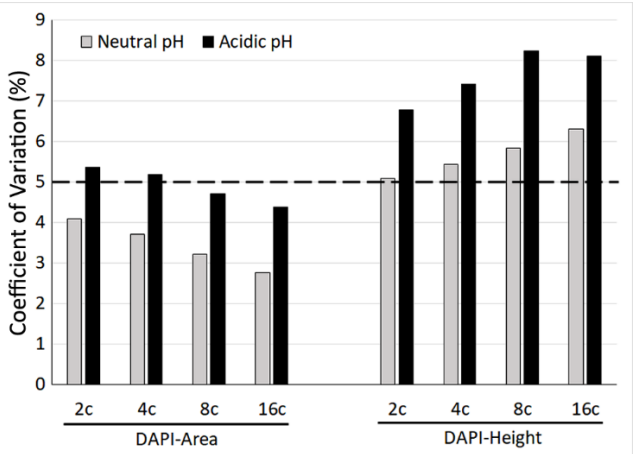

Figure 2. pH effect on Coefficient of Variation (CV%). *Phalaenopsis* leaves were chopped in GNB buffer at pH 1 (acid, dark bar) and pH 7.2 (neutral, grey bare), and nuclei were labelled with DAPI. CV (%) of endoploidy peaks have been measured on DAPI-Area signal and DAPI-Height signal (two different manners to process the fluorescence pulse: see text and Table 2). The lower the Coefficient of Variation the better the analysis.

Table 3. *Phalaenopsis* leaves have been chopped in GNB buffer at pH 1 (acid) and pH 7.2 (neutral), and labelled with DAPI. Ratios of mean fluorescence intensity of consecutive endoploidy levels were calculated for each pH condition, by using Area or Height signals (two different manners to process the fluorescence pulse: see text and Fig. 2)

	DAPI-Area		DAPI-Height	
Fluo ratio	**Neutral**	**Acid**	**Neutral**	**Acid**
4c/2c	2,01	2,01	1,89	1,83
8c/4c	2,00	1,98	1,90	1,84
16c/8c	2,00	1,98	1,87	1,79

Choice of tissues condition

GNB buffer has been tested successfully on many dried samples (Razafinarivo et al., 2012), working on angiosperms and gymnosperms (Farhat & Siljak-Yakovlev, pers. communication).

Nevertheless, some precautions have to be taken on dried samples as the peak's CV increases compared to that on fresh samples (Figure 3). Therefore, measurements may be less precise and may even need some correction factor (Razafinarivo et al., 2012).

Leaves are the material of choice for nuclei isolation. However, leaves of some species are not the best tissue because of compounds that make the preparation cloudy or/and sticky. For *Aeschynomene* for example, it is better to remove leaves and isolate nuclei from stem, which makes the preparation cleaner and more fluid (Chaintreuil et al., 2018).

Because of processes such as endoreplication and ploidy shifts, it is routine to set the cytometer with fluorescent histograms on both linear and logarithmic scales. The processes of gating are key to specifically identify the nuclei in a heterogenous, non-purified suspension. These cytometric tools are explained elsewhere (Marie & Brown, 1993; Coba de la Peña & Brown, 2001; Doležel et al., 2007).

Calculation of 2C-value

In cytometry, there are different ways to digitize the signal from detectors (photomultiplier or avalanche photodiode), namely by pulse-area or pulse-height. It cannot be trivial for neophyte to know on which signal the measurements have to be done. Pulse-height may be sensitive to nuclear form. Most of the time, it is better to choose area signal when Mean Fluorescence Intensity (MFI) need to be quantified, as it is the case for genome size. Table 3 shows the loss of linearity when measuring MFI on pulse-height signal. On the contrary, the linearity looks correct (about 2.00) by measuring on pulse-area signal, and conditions are correct for calculating 2C.

The mean of 2C-value as well as the standard deviation of the mean and its coefficient of variation (%) are calculated from measurements of samples comprising at least three to five individuals. The monoploid genome size (1Cx) is the DNA content of a monoploid genome, with chromosome base number *x*, calculated by dividing the 2C value by ploidy level (Greilhuber et al., 2005).

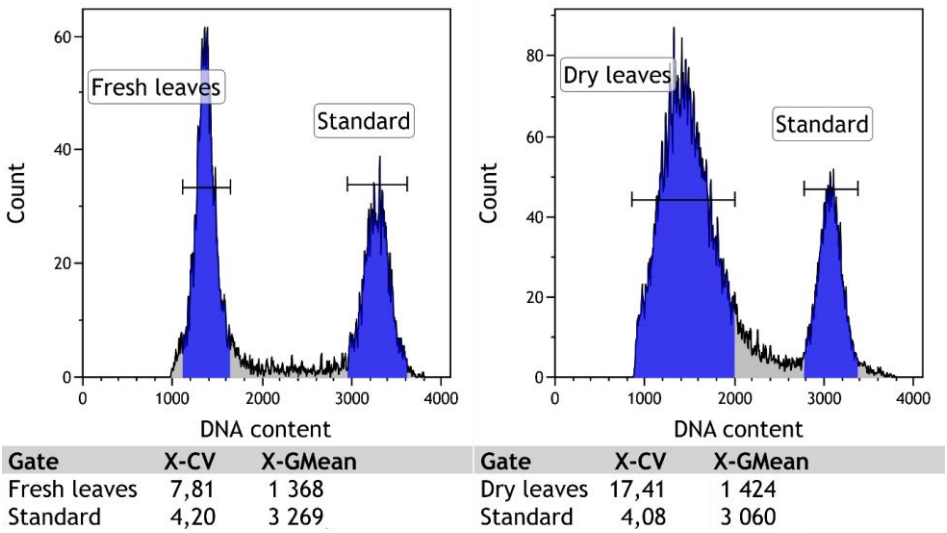

Figure 3. Genome size estimation of *Coffea pseudozanguebariae* Bridson by using fresh or dried leaves. Statistically, the DNA estimation is similar using either material, although the CV is less satisfactory with dried leaves. Occasionally, due to tanning, some correction factor may be required between estimates from fresh and dried material (Razafinarivo et al., 2012).

The ratio of fluorescence intensity (FI) of 2C-nuclei from sample and standard allows calculation of genome size of testing plant ($2C_{test}$).

$$2C_{test}(pg) = FI_{test}/FI_{standard} x\ 2C_{standard}(pg)$$

The symbol C corresponds to the holoploid nuclear genome size (the whole chromosome complement with chromosome number *n*), 1C and 2C being, respectively, the DNA contents of the haploid (*n*) and diploid (2*n*) sets of chromosomes, irrespective of ploidy level (Greilhuber et al., 2005).

2. Other uses of flow cytometry

Estimation of AT/GC%

Genome bases composition can be measured by using different dyes (Godelle et al., 1993). This calculation of AT/GC% is based on the ratio comparison between test and standard, obtained with 2 or 3 different fluorochromes: one intercalant (ethidium bromide (ET) or propidium iodide (PI)), one labelling dependent on AT composition (bisbenzimide Hoechst 33342, DAPI) and/or one labelling dependent on GC composition (chromomycine A_3 or mithramycine). It is then necessary to have a cytometer with different lasers able to excite these three categories of fluorochromes.

Optimal fluorescent emission of bisbenzimide Hoechst 33342 is obtained when it is bound to five consecutive AT. Similarly, optimal emission of Chromomycine A_3 (or Mithramycine) is obtained when these are bound to 2-3 consecutive GC. According to this stoichiometry with differential bounds, the following formula will be used to calculate bases composition:

RPI = Intensity test/Intensity standard measured with PI (or ET)

RHO = Intensity test/Intensity standard measured with Hoechst 33342

RCA = Intensity test/Intensity standard measured with Chromomycine A_3 (or Mithramycine)

$\%AT_{test} = \%AT_{standard} \times (RHO / RPI)^{1/5}$

$\%GC_{test} = \%GC_{sstandard} \times (RCA / RPI)^{1/3}$

Check: %GCtest + %ATtest = 100 (%)

This method, also applicable to other kingdoms, allows rapid measurement of base composition of genomes, complementing a systematic study of genome evolution (Siljak-Yakovlev et al., 1996).

Detection of different ploidy levels

Genome size measurement gives an estimation of the likely individual ploidy. This is very important in ecology and systematics to understand evolutional mechanisms that occur between populations within a same species or between several species. Ploidy is strictly determined by the number of chromosome complements. Concerning cytometry results, one talks of "DNA ploidy" where the ploidy of a reference plant genome is known, and then ploidy of unknown species is deducted by comparison of DNA contents (Figure 4), and not strictly by counting chromosomes. The principle interest of this cytometric approach is the ability to make hundreds of analyses in one day (or thousands of measurements with automats and plate-readers), without the difficulties of cytogenetics, which nevertheless remain crucial to validate this estimation.

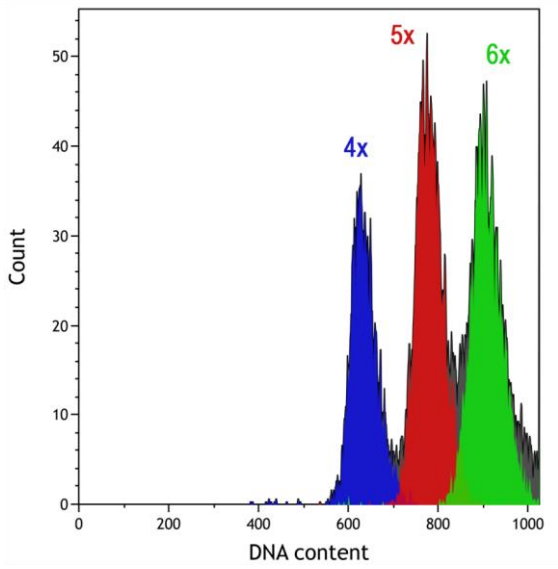

Figure 4. The leaves of three *Cenchrus ciliaris* plants corresponding to populations with different ploidy levels were chopped simultaneously (without any internal standard plant) and isolated nuclei were labelled with 50 µg/ml PI. Each peak presents one of three ploidy level 4x, 5x and 6x.

This method allows fast estimation of ploidy from batches of plants during varietal creation, which represents a time-saving trick for agronomy and seed industry. It is also necessary to check the ploidy of the reference specimen used in laboratory research. In vitro manipulation of transformation can induce polyploid or aneuploid individuals, which are generally better to discard.

Analysis of cell cycle

Cell cycle of plant cells has similarity with other eukaryotes. It comprises a G1 phase of cell growth, S phase of DNA synthesis, G2 phase of mitosis preparation and M phase of mitosis.

Measurement of plant nuclear DNA content is optimal when free of the cell wall. Isolated protoplasts can be labelled and analysed by cytometry. Nevertheless, optic interferences of organelles, especially plastids with DNA and high refractive index, disturb cell cycle measurements, increasing peak CV. Cell cycles are then more difficult to interpret than in other eukaryotes cells or as in isolated plant nuclei.

So the easy approach, fast and precise, is to pull nuclei out of cells by chopping tissue, like during of genome size estimation. Obviously, this protocol is not appropriate when it is necessary to analyse the cycle along with immuno labelling or fluorescent proteins (GFP family for example) in multicolour analysis, except if these additional markers are strictly localized in the nuclei.

Histogram interpretation of nuclear DNA content is relatively simple in proliferating cell culture. Cellular proliferation in plants usually takes place in meristems, surrounded by differentiating cells. This proliferation is not uniform, time of cycle being variable between different zones. Some cells can be quiescent at different ploidy levels (2C, 4C, 8C, etc.), compared to a majority of cells in endoreplication. The 4C nuclei, for instance, may be actively cycling G2 or may instead be from differentiating cells.

Case of Endoreplication

A feature in plants cell cycle is endoreplication: a modified cell cycle without mitosis phase, which leads to cells with high ploidy level. In the same tissue or organ, plant cells are able to have different ploidy levels called polysomaty. However, these endoreplication cycles (or endopolyploidy) are strictly regulated for a given species, a given organ, and a given development step. This endoreplication process is essential for plant development, and a modification in ploidy level distribution can induce atypical development (Raynaud et al., 2005). Endoreplication is also involved in fruit development (Pirrello et al., 2014; 2018), tissues for stock, and in some cases of pathogen invasion, like gall from roots nematode, or callus formation. This endoreplicaton is exponential and uniform in nuclear DNA content, as if replication and repairs were perfectly maintained: the CV of peaks 2C, 4C, 8C, etc. are generally identical throughout the DNA histogram.

How to quantify endoreplication? Barow & Meister (2003) have introduced “cycle value”, using the number of cycles per nuclei, a calculation taken under “endoreplication index”. Nevertheless, their formula includes 2C and 4C nuclei. Instead, we propose 2 parameters corresponding to 2 different questions:

- How many nuclei from tissue undergoes endoreplication
  “Endoreplication switch frequency” = ES = f > 4C, where f is nuclei frequency (%)
- What is the level of this endoreplication
  “Endocycle index” = E = $[(1 \times f_{8C}) + (2 \times f_{16C}) + (3 \times f_{32C}) + (4 \times f_{64C}) + \ldots] / (f > 4C)$.

This focusses on the number of cycles per nuclei beyond 4C. This last parameter is independent of 2C and 4C nuclei.

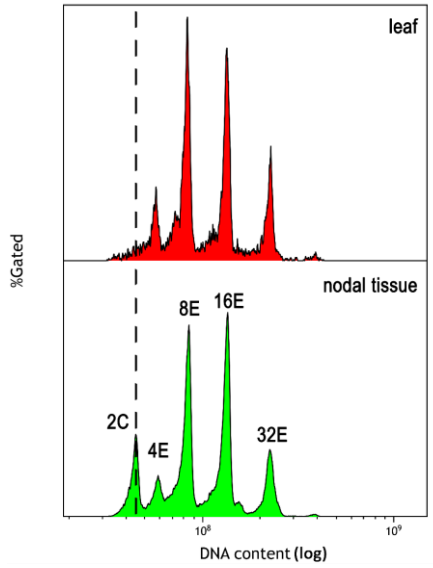

Figure 5. DNA histograms from *Vanilla planifolia* somatic tissues. Here, the X-axis is logarithmic. Note that euploid nuclei are sometimes difficult to assess in leaf, complicating the estimations of genome-size and of the endoreplication process itself. Contrastingly, in nodal tissue, the 2C nuclei, essential for assessing genome size, constituted exceptionally a fifth of this sample of orchid nuclei. Populations of larger nuclei were also evident: 4E, 8E, 16E, 32E, and a trace of 64E. E is the quantity of DNA association with a single genome undergoing endoreplication.

Surprisingly, a distinct endoreplication process has been observed in *Vanilla* species and other orchids. Cytometry data shows that endoreplication is not exponential but partial. In *Vanilla planifolia*, replication observed in each endocycle is not 2.00 but ~ 1.45, 1.6, 1.7, and 1.8 (Brown et al., 2017).

The cumulative effect of this "strict partial endoreplication" leads to a loss of 41% of expected DNA content in 32C nuclei. Although this process is observed in many populations, varieties and species (*V. tahitensis*, *V. pompona*, *V. planifolia*) and in other orchid genera (*Orchis*, *Ophrys*, *Dactylorhiza*), nothing is known yet about mechanism and molecular determinants, and endoreplication is perfectly linear in some other orchids (*Phalaenopsis*, *Cymbidium*).

Another particularity is the difficulty to determine the first 2C peak during this process: it may be absent from specific tissue, or show only a trace that is overlooked. Leaves are often the preferential tissue to chop for genome size, ploidy and endoreplication, but Figure 5 shows that 2C peak is missing in *Vanilla* leaves most of the time. This had led to some errors of genome size estimation in literature. It is wise to test other tissue for chopping, such as meristematic nodal tissue for monocotyledon families such as the orchids (Figure 5).

Flow cytometric seed screening (FCSS) as tool for reconstruction of reproductive pathway

Flow cytometric seed screening developed by Matzk et al. (2000) is used to reveal the pathway of reproduction (pure sexual and obligate or facultative apomixis) and to identify the seed origin and ploidy level of gametes that participated in the embryo and/or endosperm fertilization process.

Reproduction pathway analysis is based on the relationship between ploidy levels of embryo and endosperm nuclei from mature seeds. The entire seeds (or with tegument removed) could be chopped with fresh leaf of an appropriate standard in cold Gif Nuclear Buffer. The remaining protocol steps are the same as previously described for assessment of genome size and ploidy level, using both linear and logarithmic DNA scales.

In somefield trials, the screen of mature seeds by flow cytometry produced more information about the reproductive behavior of individual plants than any other available test. It is very useful in both basic research and plant breeding (screening of descendants). Thereby, this method for the detection of the mode of reproduction has almost completely replaced laborious and long embryological studies; it has become the principal tool in investigations concerning polyploidy, apomixis and breeding events (Hajrudinovic et al., 2015).

To illustrate the use of this technique we present part of results from our study on *Sorbus* genus from Bosnia and Herzegovina (Hajrudinovic et al., 2015). In sympatric populations of diploid *Sorbus aria* and tetraploid apomictic *S. austriaca* it was observed that the coexistence of apomictic tetraploids and sexual diploids drives the production of novel polyploidy cytotypes (3x) with predominantly apomictic reproductive modes (Figure 6).

The analysis of different ploidies of embryos and endosperms reflected different pathways of seed origin. The seeds of diploid mothers were of sexual origin with diploid embryo and triploid endosperm (Figure 6A). Triploid mothers produced the seeds of apomictic origin where the embryo was always triploid and endosperm ploidies ranged from 8x to 11x which indicate different pathways of endosperm formation (Figure 6B and C). Apomictically formed seeds in tetraploid *S. austriaca* presented 4x embryos and endosperm of 10x, 11 or 12x (Figure 6D).

Nuclei/Chromosome sorting

One way to obtain nuclei from different cell cycle phases, with a higher purity than chemical synchronisation, is to isolate them by chopping and sorting by cytometry. The yield will be lower but sufficient to do downstream molecular biology experiments (Pirrello et al., 2014) or imaging (Bourdon et al., 2012; Brown et al., 2017).

Beyond nuclei, cytometry technology allows chromosome sorting. Flow cytometry has been used to sort chromosomes in at least 24 plant species (Doležel et al., 2014). Staining chromosomal DNA with PI, DAPI or Hoechst 333242 results in a distribution of fluorescence signal intensity, in which, ideally each chromosome can be recognized by a different peak. In reality, some peaks are too closed to each other and cannot be distinguished. A second parameter is used to overcome peak overlay:

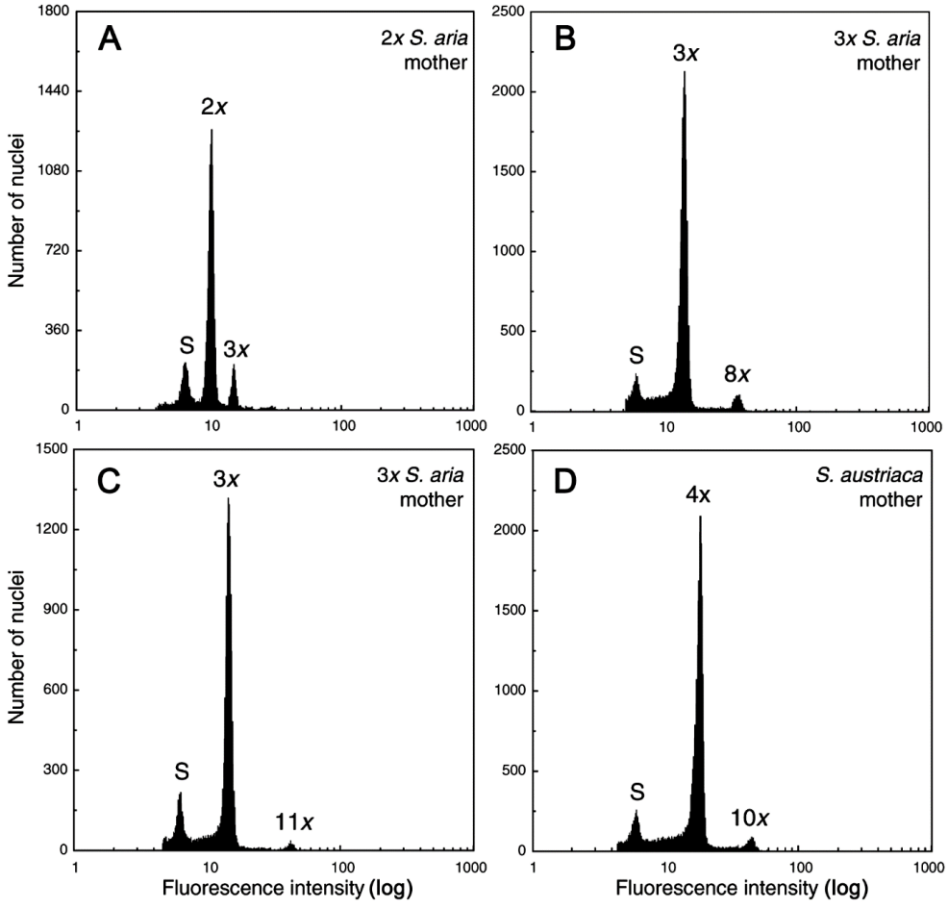

Figure 6. An example of Flow Cytometric Seed Screening (FCSS). This diploid *Sorbus aria* mother produced only seeds of sexual origin (A). The triploid *S. aria* (B,C) and the tetraploid *S. austriaca* (D) mothers produced mainly seeds of apomictic origin. The first fluorescence peak corresponds to the internal standard (S, *Oryza sativa* ssp. *japonica* 'Nipponbare'), the second to the embryo and the third to the endosperm (from Hajrudinovic et al. 2015). Here, the X-axis is logarithmic.

FISHIS (FISH in suspension) largely described by the Doležel team (Doležel et al., 2014; Vránaet al., 2016). Plant chromosome sorting by flow cytometry has been used for a range of applications, including cytogenetic analysis, physical and genetic mapping and whole genome sequencing.

Conclusions

Flow cytometry is widely used across biological sciences, including microbiology. However, this technology has been somewhat restricted in plant sciences due to the impediment of the cell wall. Nevertheless, making cell cycle analysis, assessing polyploidy, making ploidy checking easier, and characterising the variability of populations' genome size in ecology or systematics, are useful and topical methods. Although quantitative fluorescent imaging has improved, flow cytometry remains a powerful tool for accurate simultaneous quantification of multiple parameters of cellular variables. The two approaches, cytometry and microscopy, are complementary and can be combined (Bourge et al., 2015; Pirrello et al., 2014).

New methods and applications remain to be mastered. Small portable cytometers are available and could be used for field cytometry of plants as in microbiology, epidemiology or hydrobiology. Plant biologists, and in general all biologists would do well to follow the developments and applications of cytometry.

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