Chapter 14

The Application of Flow Cytometry for Estimating Genome Size and Ploidy Level in Plants

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Abstract

Over the years, the amount of DNA in a nucleus (genome size) has been estimated using a variety of methods, but increasingly, flow cytometry (FCM) has become the method of choice. The popularity of this technique lies in the ease of sample preparation and in the large number of particles (i.e., nuclei) that can be analyzed in a very short period of time. This chapter presents a step-by-step guide to estimating the nuclear DNA content of plant nuclei using FCM. Attempting to serve as a tool for daily laboratory practice, we list, in detail, the equipment required, specific reagents, and buffers needed, as well as the most frequently used protocols to carry out nuclei isolation. In addition, solutions to the most common problems that users may encounter when working with plant material and troubleshooting advice are provided. Finally, information about the correct terminology to use and the importance of obtaining chromosome counts to avoid cytological misinterpretations of the FCM data are discussed.

Key words Chromosome number, DAPI, DNA ploidy level, Genome size, Flow cytometry, Flow histogram, *C*-value, PI, Plant nuclei isolation, Relative fluorescence

1 Introduction

The total amount of DNA in the nucleus of an organism is generally referred to as the genome size, and it is measured either in picograms (pg; i.e., 1×10^{-9} g) or megabase pairs (Mbp, with 1 pg = 978 Mbp, [1]). People started to investigate genome size in plants even before the structure of DNA was worked out, with the first plant to have its genome size estimated being *Lilium longiflorum* in 1951 [2]. Since then the genome sizes of over 8,500 species have been estimated [3] with the data being used not only for practical applications (e.g., how much will it cost to sequence a genome? how many clones are needed for making BAC libraries?) but also for providing valuable insights into many biological fields, including evolution, systematics, ecology, population genetics, and plant breeding (*reviewed in* refs. [4–7]).

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Over the years, several methods have been used to estimate genome sizes in plants (e.g., Feulgen densitometry, reassociation kinetics). Nevertheless, in recent years, due to a variety of reasons, flow cytometry (FCM) has become the method of choice [8]. Briefly the method involves three steps: (1) a sample of plant tissue is chopped in a suitable buffer to release the nuclei; (2) the nuclei are stained with a fluorochrome that binds quantitatively to the DNA, so the bigger the genome, the more stain that is bound to the DNA; and (3) the nuclei are passed through a flow cytometer which measures the amount of stain bound to each nucleus (Fig. 1a). By preparing a combined sample which includes a plant species with a known DNA amount (reference standard), the

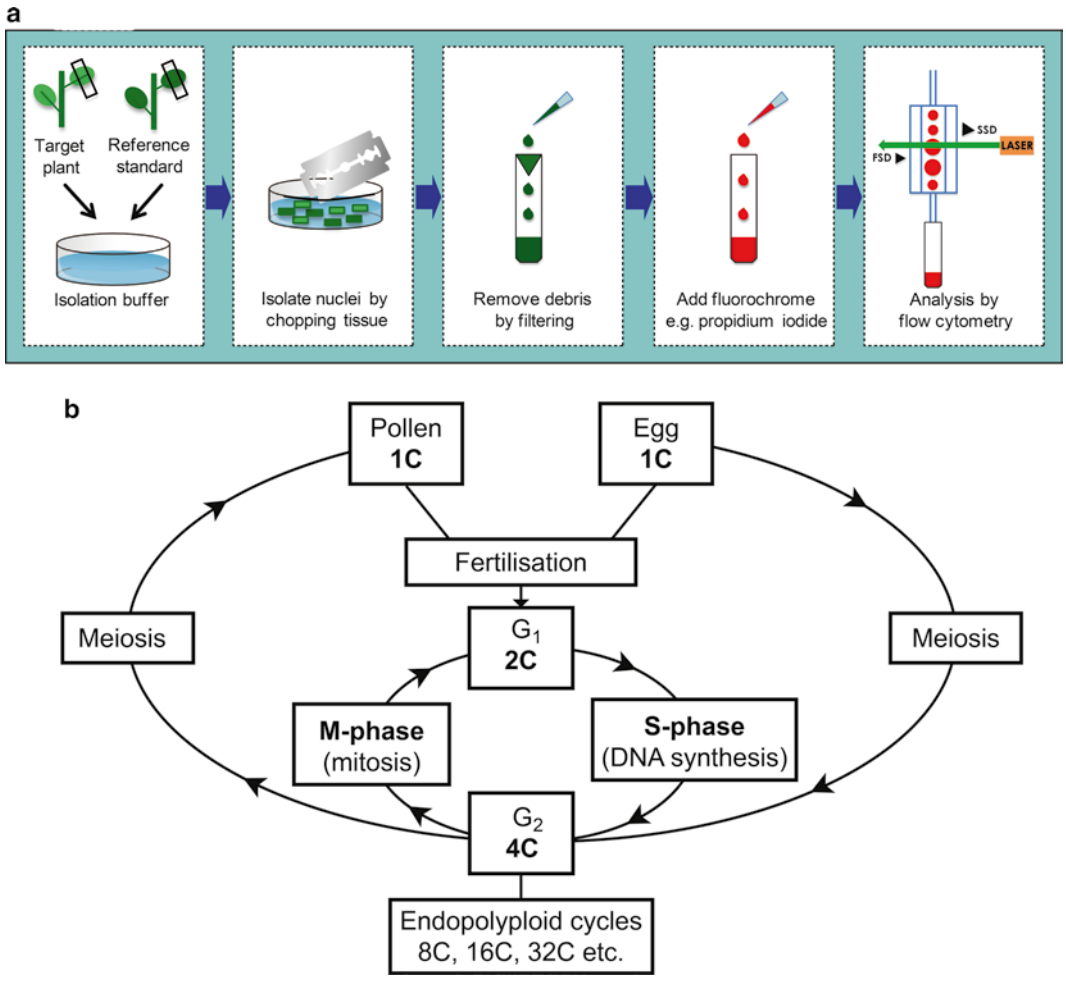

Fig. 1 (**a**) The basic steps involved in the estimation of genome size and ploidy level by flow cytometry. (**b**) Changes in the holoploid *C*-value at different stages of the cell cycle and following meiosis and endopolyploidy (N.B. cells which undergo endopolyploidy (i.e., DNA synthesis not accompanied by mitosis) will have *C*-values greater than 4*C* (i.e., 8*C*, 16*C*, 32*C*, etc., depending on the number of rounds of DNA replication))

relative amount of fluorescence from the target plant can be converted into an absolute genome size. It is important to realize that FCM only gives information about the relative or absolute DNA amount of the isolated nuclei; it does not provide cytological information. Yet without such information, interpretations of the chromosome number and/or ploidy level of the species can be flawed. Subheading 1.2 below highlights the importance of obtaining such cytological data and the pitfalls and errors that can arise without it.

FCM can also be used to estimate the ploidy level of a plant based on comparing the genome size of the target species either with the genome size of a specimen of known ploidy (i.e., determined karyologically) or with an internal standard (in that case the reference standard must be kept constant throughout the experiment and the ploidy level of at least one target sample should be karyologically determined). However, in such cases, the ploidy level is referred to as the "DNA ploidy" to distinguish it from studies where ploidy level has been determined karyologically [9]. Such approaches are now being increasingly used to survey the diversity of cytotypes across plant populations and have uncovered a surprising diversity of hitherto unsuspected ploidy variation in some species [4–6].

This chapter outlines the general method used to estimate genome size and/or determine the DNA ploidy level in plants using FCM (Subheadings 2, 3, and 4). However, given the immense diversity of plants in terms of their morphology (e.g., woody, succulent, herbaceous) and biochemistry (e.g., presence of pigments, tannins, phenolics), problems may well be encountered. The majority of these arise mainly from the interaction between chemicals present in the cell cytoplasm and the binding of the fluorochrome to the DNA [10–16] leading to erroneous results. Thus, this chapter also outlines some of the more commonly encountered problems and ways in which the poor results might be improved to overcome these issues.

In addition to the information given here, it is also suggested that the FLOWer database [17, 18] is consulted as this provides an extensive list of papers which have used FCM to estimate genome size in plants. It is worth checking whether the particular genus of interest has previously been studied by FCM and, if so, whether any particular modifications were made to the buffers and protocols used, to overcome specific problems associated with the particular genus being analyzed. In addition, genome size databases may also be useful to check in order to get some idea about the range of genome sizes one might expect for a given taxa. Examples include the Plant DNA C-values Database [3] which contains data for all the major groups of land plants and three algal lineages, while the more focused database containing genome size data for Asteraceae (GSAD—19) is ideal for specific studies focused on this family of angiosperms. Such prior information can save a lot of time and frustration!

1.1 Terminology Used for Genome Size Studies

Given that the amount of DNA varies throughout the cell cycle (i.e., G_2 nuclei have twice the DNA amount as G_1 nuclei) (Fig. 1b), and following meiosis and endopolyploidy (somatic polyploidy), considerable confusion can arise when discussing genome sizes. To overcome such issues, Greilhuber et al. [20] proposed the following terminology which has now been widely adopted.

1. *Holoploid 1C-value* (abbreviated to 1*C*-value) refers to the amount of DNA in the unreplicated gametic nucleus (e.g., pollen or egg cell of angiosperms) regardless of the ploidy level of the cell. The 2*C*-value represents the amount of DNA in a somatic cell at the G_1 stage of the cell cycle, while the 4*C*-value is the amount in a somatic cell at the G_2 stage, following DNA synthesis (*S*-phase) (*see* Fig. 1b).
2. *Monoploid 1C-value* (abbreviated to 1*Cx*-value) refers to the amount of DNA in the unreplicated monoploid (x) chromosome set. For a diploid organism where $2n = 2x$, the 1*C* and 1*Cx* values are the same; however, for a polyploid organism, the 1*Cx* is always smaller than the 1*C*-value (e.g., for a tetraploid where $2n = 4x$, then $1Cx = 1/2\ 1C$, whereas for a hexaploid where $2n = 6x$, then $1Cx = 1/3\ 1C$).

1.2 The Importance of Cytological Data for Genome Size Studies

As noted above, FCM only measures the total amount of DNA in the nucleus and gives no specific information about the chromosome number or ploidy level of the plant analyzed (although this can be deduced in certain cases as outlined in Subheading 3.2.3). Despite this, many studies report a ploidy level or chromosome number for the analyzed plant which has either been taken from the literature or based on comparisons of DNA amounts found in related species. This is fine in a stable cytological system where there is little variation in chromosome number and size between species. However, in plants, such situations are probably the exception rather than the rule, even between closely related taxa, as many genera show considerable cytological diversity—for example, (1) polyploidy, both within and between species, is frequent, (2) large divergences in genome size among closely related species with the same ploidy have been reported, and (3) increases in ploidy level or chromosome number are not necessarily accompanied by proportional changes in DNA amount.

Examples of problems and misinterpretations of genome size that can arise through assuming the ploidy level and/or chromosome number of a species have been discussed by Suda et al. [9]. Below are a few examples to illustrate the pitfalls that can arise when karyological information is not obtained in parallel with genome size data.

1. In species with a constant chromosome number but a big range in size, an absence of chromosome data could lead to the erroneous suggestion that polyploids may be present to explain the large range of genome sizes encountered. This is illustrated by the genus *Cypripedium* (Orchidaceae) where

most species have a chromosome count of $2n = 20$ but genome size has been shown to vary over tenfold between species ($1C = 4.1$–43.1 pg) [21] (*see* Fig. 2a, b). A similar situation has also been reported in the genus *Artemisia* (Asteraceae), where the diploid species *A. annua* with $2n = 18$ chromosomes has a $1C$-value of 1.75 pg [22], while *A. leucodes*, with the same chromosome number, has a $1C = 7.70$ pg [23]. Without doing a chromosome count, one could easily assume that *A. leucodes* was a polyploid given such differences in genome size.

2. Erroneous assumptions of ploidy level in a studied species can arise when increases in chromosome number via polyploidy have not been accompanied by proportional increases in genome size. This is likely to be a common problem since genome downsizing following polyploidy is frequently encountered in angiosperms [24]. In extreme cases, species with higher ploidy levels may have the same or lower genome sizes than related species of lower ploidy. An example of this is provided by the genus *Physaria* (Brassicaceae) where the high

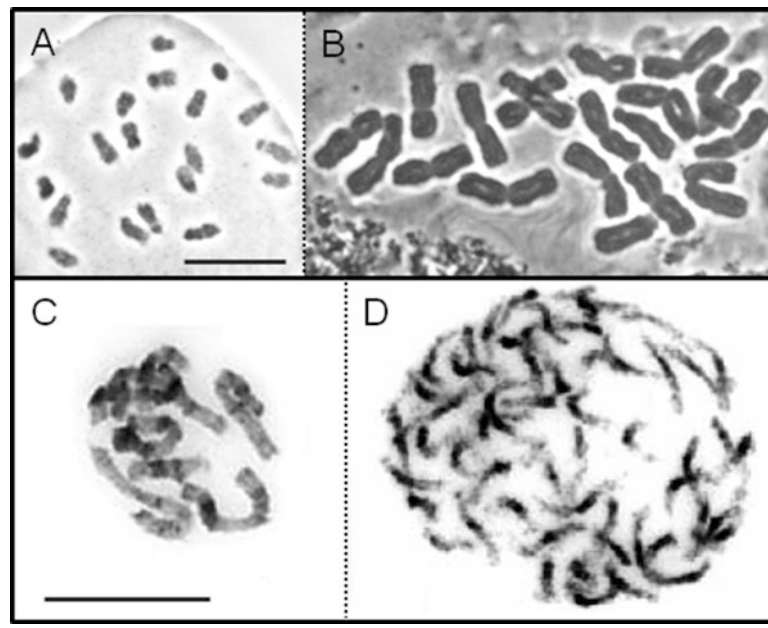

Fig. 2 Examples where chromosome size and number in related species do not correlate with genome size. Chromosomes of (**a**) *Cypripedium molle* ($1C = 4.1$ pg) and (**b**) *C. calceolus* ($1C = 32.4$ pg) taken at the same magnification showing an eightfold range in genome size but a constant chromosome number of $2n = 20$. Image reproduced with permission from ref. 21 (scale bar = 10 μm). Chromosomes of (**c**) diploid *Physaria bellii* ($2n = 2x = 8$; $1C = 2.34$ pg) compared with those from (**d**) the high polyploid *P. didymocarpa* ($2n = 14x = 56$; $1C$ 2.23 pg). Both species have similar genome sizes but very different chromosome numbers and sizes. Image reproduced with permission from ref. 25 (scale bar = 5 μm)

polyploid *P. didymocarpa* with $2n = 14x = 56$ actually has a smaller genome ($1C = 2.23$ pg) than a related diploid *P. bellii* ($2n = 2x = 8$) with $1C = 2.34$ pg [25] (Fig. 2c, d).

3. Nonproportional changes in DNA content have also been reported in different cytotypes of the same species. Once again, this has the potential to lead to erroneous deductions of ploidy level based on genome size data alone. Both increases and decreases in the size of monoploid genomes have been reported with increasing ploidy levels. For example, in *Larrea tridentata* (Zygophyllaceae), the hexaploid cytotype was reported to have just 1.25 times more DNA than the tetraploid [26]. Without chromosome data to support this, it is possible that the hexaploid could have been misidentified as a pentaploid, based on DNA amount alone.

In addition to these examples, it is also important to note that many chromosomal changes and variations (e.g., aneuploidy, chromosome duplications and deletions, sex and supernumerary chromosomes, supernumerary segments) can arise which are detectable as changes in DNA amount. Without identifying these through cytological analysis, further misinterpretations of the data may arise.

Overall, these examples serve to illustrate how serious mistakes can be made in the absence of karyological information. Thus, it is strongly recommended that chromosome counts are made of the plant used for genome size estimation. If this is not possible, then the ploidy level should always be referred to as the "DNA ploidy level" as discussed by Suda et al. [9].

2 Materials

Detailed information about plant tissues, reagents, composition of the isolation buffers, as well as the technical equipment needed to carry out genome size and ploidy estimations using FCM are described below.

2.1 Plant Tissue and Reference Standards

Of the potential plant tissues suitable for genome size estimation, leaf tissue is preferred by researchers because it generally gives the best results. Nevertheless, other plant tissues such as petals, stems (including petioles), roots, pollen grains (including pollinia), and seeds (dried or fresh) [27–29] can be considered as viable alternatives for genome size estimations. When fresh plant tissues are selected, they should be as fresh as possible and collected from young and actively growing parts of the plant as such material is likely to give the best results. Old and senescent tissues will probably result in higher levels of background signal and may contain high proportions of nuclei at the G_2 phase of mitosis.

In addition, silica-dried leaves and herbarium vouchers may be used to estimate DNA ploidy levels [30]. However, given that

DNA deterioration is likely to occur in such samples, the material is not considered suitable for high-quality estimations of genome size in absolute units. Nevertheless, recent studies have suggested that maybe even dried material can be used for genome size estimations if it has been appropriately desiccated [31].

As an alternative to desiccation, a recent publication has demonstrated the suitability of glycerol-preserved nuclei for estimating genome size in absolute units for material up to at least a few weeks old [32]. This method has been designed for field research, and although it still has a few limitations (i.e., only high-quality results are obtained when samples are kept in ice-cold buffer), it demonstrates the efforts that researchers in this discipline may go to in order to overcome problems associated with the current limited time scale available to analyze large batches of fresh material without compromising quality of the results.

Concerning reference standards, we recommend that several species, covering a broad range of genome sizes, are kept growing in the laboratory to enable the most appropriate standard to be selected for each particular analysis. Many species have been used [17] but we summarize some of the most popular ones in Table 1 which work well with FCM.

Table 1
Several reference standard species recommended for genome size estimation

Plant species	1*C* DNA content (pg)	Reference
Oryza sativa L. "IR-36"	0.50	[49]
Raphanus sativus L. "Saxa"	0.55	[50]
Solanum lycopersicum L. "Stupiké polní rané"	0.98	[50]
Vigna radiata (L.) R.Wilczek "Berken"	1.20	[49]
Glycine max Merr. "Polanka"	1.25	[51]
Petunia hybrida Vilm. "PxPc6"	1.42	[52]
Petroselinum crispum (Mill.) Nyman ex A.W.Hill "Champion Moss Curled"	2.22	[53]
Zea mays L. "CE-777"	2.71	[54]
Pisum sativum L. "Express Long"	4.18	[52]
Pisum sativum L. "Ctirad"	4.54	[55]
Pisum sativum L. "Minerva Maple"	4.86	[49]
Secale cereale L. "Daňkovské"	8.09	[55]
Vicia faba L. "Inovec"	13.45	[50]
Allium cepa L. "Ailsa Craig"	16.77	[49]
Allium cepa L. "Alice"	17.42	[55]

2.2 Equipment Needed

1. Set of pipettes with disposable tips (100 μL, 1 mL).
2. Razor blades (double-edged) or scalpel with replaceable blades. A razor blade holder or alternative protective device (e.g., cork or silicon bung) is also recommended.
3. Plastic petri dishes (c. 5–6 cm diameter).
4. Disposable nylon mesh filters (30–42 μm pore size; e.g., Partec, cat. no. 04-0042-2316). Alternatively, regular nylon mesh cut into squares and fitted on disposable tips can be used.
5. Sample tubes suitable for the particular flow cytometer being used (check manufacturer's specifications in each case).
6. 1.5 mL tubes.
7. Sample tube racks.
8. Plastic and/or expanded polystyrene containers to fill with ice.
9. Latex, nitrile, or vinyl gloves. Safety goggles and lab coat.
10. Centrifuge fitted with a rotor suitable for 1.5 mL tubes.
11. Fridge and freezer.
12. Flow cytometer fitted with the light source suitable for excitation of the DNA fluorochrome used in the study (check fluorochrome's excitation and emission spectra to select the suitable excitation sources following the manufacturer's recommendations).
13. Analytical software for evaluation of flow cytometric data (usually provided by the manufacturer of the flow cytometer).
14. Fume cupboard to carry out nuclei isolation using buffers supplemented with either β-mercaptoethanol or DTT (*see* **Note 1**).
15. Cleaning and decontamination solutions for flow systems. Domestic sodium hypochlorite (bleach) diluted 1:5 in distilled water.
16. Calibration particles: fluorescent beads [e.g., Partec, cat. no. 05-4006 (green), 05-4020 (UV)].

2.3 Reagents

2.3.1 Fluorochromes

1. PI (propidium iodide—*see* **Note 2**): Prepare a stock solution of 1 mg/mL and filter through a 0.22 μm filter. Store in 1 mL aliquots at −20 °C (*see* **Note 1**). The working concentration of PI is usually 50 μg/mL.
2. DAPI (4′,6-diamidino-2-phenylindole—*see* **Note 2**): Prepare stock solution of 0.1 mg/mL and filter through a 0.22 μm filter. Store in 1 mL aliquots at −20 °C (*see* **Note 1**). The working concentration of DAPI is normally 4 μg/mL.
3. SYBR Green I (*see* **Note 2**): The stock solution provided by the manufacturer is usually 10,000× concentrate, and manufacturers recommend a working concentration of 10×. The stock should first be diluted 100-fold in DMSO (dimethyl sulfoxide—*see* **Note 1**) to give a diluted solution of 100× (e.g.,

50 μL SYBR I in 4.95 mL of DMSO). This 100× solution can be stored in 5 mL aliquots at −20 °C. For use, the appropriate volume of this diluted 100× solution is added to the nuclei isolation buffer to give a final working concentration of 10×.

2.3.2 Isolation Buffers

Isolation buffers must be prepared using either single- or double-distilled water, filtered through a 0.22 μm filter to remove suspended particles, and stored as specified. Most of the buffers remain stable for up to 3 months if appropriately stored (*see* **Notes 3** and **4**). As indicated below, some buffers can be stored for longer by freezing them in aliquots at −20 °C. However, if this is done, then once thawed, the buffer should not be refrozen. The pH of the buffers is adjusted either with 1 M NaOH or 1 N HCl (*see* **Note 5**). Further information about the roles of the different buffer components is given in **Notes 6** and **7**, while additional details of the protocols can be found in the original references cited for each buffer.

1. *LB01 buffer* [33]: 15 mM Tris, 2 mM Na_2EDTA, 0.5 mM spermine.4HCl, 80 mM KCl, 20 mM NaCl, 0.1 % (v/v) Triton X-100. Adjust to pH 7.5. Add β-mercaptoethanol to give a final concentration of 15 mM (*see* **Note 1**). Store the buffer either at 4 °C if used regularly or at −20 °C in 10 mL aliquots.
2. *Tris $MgCl_2$ buffer* [34]: 200 mM Tris, 4 mM $MgCl_2$, 0.5 % (v/v) Triton X-100. Adjust pH to 7.5 and store at 4 °C.
3. *Galbraith buffer* [35]: 45 mM $MgCl_2$, 20 mM MOPS (*see* **Note 1**), 30 mM sodium citrate, 0.1 % (v/v) Triton X-100. Adjust pH to 7.0. Store the buffer either at 4 °C if used regularly or at −20 °C in 10 mL aliquots.
4. *General purpose buffer* [36]: 0.5 mM spermine.4HCl, 30 mM sodium citrate, 20 mM MOPS (*see* **Note 1**), 80 mM KCl, 20 mM NaCl, 0.5 % (v/v) Triton X-100. Adjust to pH 7.0. Store the buffer either at 4 °C if used regularly or at −20 °C in 10 mL aliquots.
5. *Woody plant buffer* [36]: 200 mM Tris, 4 mM $MgCl_2$, 2 mM Na_2EDTA, 86 mM NaCl, 10 mM sodium metabisulfite, 1 % PVP-10 (*see* **Note 7**), 1 % (v/v) Triton X-100. Adjust to pH 7.5. Store the buffer either at 4 °C if used regularly or at −20 °C in 10 mL aliquots.
6. *$MgSO_4$ buffer* [37]: 9.53 mM $MgSO_4$, 47.67 mM KCl, 4.77 mM HEPES, 6.48 mM DTT (*see* **Note 1**), 0.25 % (v/v) Triton X-100. Adjust to pH 8.0. Store the buffer either at 4 °C if used regularly or at −20 °C in 10 mL aliquots.
7. *Bino's buffer* [38]: 200 mM mannitol, 10 mM MOPS (*see* **Note 1**), 0.05 % (v/v) Triton X-100, 10 mM KCl, 10 mM NaCl, 2.5 mM DTT (*see* **Note 1**), 10 mM spermine.4HCl, 2.5 mM Na_2EDTA, 0.05 % (w/v) sodium azide (*see* **Note 1**). Adjust to pH 5.8 and store at 4 °C.

8. *De Laat's buffer* [39]: 15 mM HEPES, 1 mM Na_2EDTA, 0.2 % (v/v) Triton X-100, 80 mM KCl, 20 mM NaCl, 15 mM DTT (*see* **Note 1**), 0.5 mM spermine.4HCl, 300 mM sucrose. Adjust to pH 7.0 and store at 4 °C.
9. *Ebihara's buffer* [40]: 50 mM Na_2SO_3, 50 mM Tris, 40 mg/mL PVP-40 (*see* **Note 7**), 140 mM β-mercaptoethanol (*see* **Note 1**). Adjust to pH 7.5 and store at 4 °C.
10. *Seed buffer* [41]: 5 mM $MgCl_2$, 85 mM NaCl, 100 mM Tris, 0.1 % Triton X-100. Adjust to pH 7.0 and store at 4 °C (*see* **Note 8**).
11. *Otto buffer* [42]: Otto I: 100 mM citric acid monohydrate, 0.5 % (v/v) Tween 20 (*see* **Note 9**). Store at 4 °C. Otto II: 400 mM Na_2HPO_4 (*see* **Note 10**). Store at room temperature.
    The fluorochrome (DAPI or PI; see above) can be added to Otto II before adjusting the final volume of the stock solution. If this is done, the buffer should be stored in the dark at room temperature. Alternatively the fluorochrome can be added directly to the sample at **step 10** of Subheading 3.1.2 or **step 7** of Subheading 3.1.3.
12. *Baranyi's buffer* [43]: Baranyi solution I: 100 mM citric acid monohydrate, 0.5 % (v/v) Triton X-100. Store at 4 °C.
    Baranyi solution II: 400 mM Na_2HPO_4, 10 mM sodium citrate, 25 mM sodium sulfate. Store at room temperature.
    The fluorochrome (DAPI or PI; see above) can be added to Baranyi solution II before adjusting the final volume of the stock solution. If this is done, the buffer should be stored in the dark at room temperature. Alternatively the fluorochrome can be added directly to the sample at **step 10** of Subheading 3.1.2 or **step 7** of Subheading 3.1.3.
13. *Mishiba's buffer* [44]: Solution A: (see recipe for Galbraith buffer, i.e., buffer 3 above).
    Solution B: 10 mM Tris, 50 mM sodium citrate, 2 mM $MgCl_2$, 1 % PVP-40 (original recipe used PVP K-30—*see* **Note 7**), 0.1 % (v/v) Triton X-100, 18 mM β-mercaptoethanol (*see* **Note 1**). Adjust to pH 7.5. Store at 4 °C.

3 Methods

3.1 Isolation of Plant Nuclei

Nuclei suspensions can be prepared according to either the one-step protocol (Subheading 3.1.1) or the two-step protocol (Subheading 3.1.2). The one-step protocol works with most plant species and with buffers 1–10 (Subheading 2.3.2). However, for some plant groups, the two-step protocol using buffers 11 or 12 (Subheading 2.3.2) will provide histograms with much higher-quality peaks. A simplified version of the two-step protocol using buffers 11, 12, and 13 (Subheading 2.3.2) is given in Subheading 3.1.3.

We recommend (unless specified otherwise) working under cold conditions (i.e., keep all solutions, buffers, and prepared samples waiting for analysis on ice, and do the chopping step in a petri dish resting on a bed of ice). Together this helps to inhibit the negative effect of many cytosolic compounds that may be present (e.g., DNase, phenolics, tannins), and it can be especially helpful when working with recalcitrant samples.

3.1.1 Isolation of Plant Nuclei Using the One-Step Protocol

1. Place a small amount of the selected plant tissue (usually about 1 cm^2 or 20 mg) in a 6 cm petri dish (*see* **Note 11**).
2. Add 1 mL of ice-cold isolation buffer (*see* Subheading 2.3.2, buffers 1–10) to the petri dish (*see* **Note 12**).
3. Chop the tissues in the buffer using a new razor blade or sharp scalpel (*see* **Note 13**).
4. Add another 1 mL of the same ice-cold buffer as used in **step 2** (*see* **Note 14**).
5. Mix the crude suspension by gently shaking the petri dish.
6. Filter the homogenate through a 30–42 μm nylon mesh filter into a labelled flow cytometry tube (*see* **Note 15**). The chopping and filtration processes might result in a reduction in the final volume, especially when working with dried samples. To reduce any critical effect, (1) dried samples can be presoaked in buffer for up to 5–10 min, and (2) filters can also be soaked in buffer prior to filtration.
7. Add the appropriate volume of fluorochrome (Subheading 2.3.1) to the nuclei suspension and vortex gently. For a typical sample which is c. 2 mL, the amount of stock PI added is 100 μL, while for DAPI, 80 μL should be added (*see* **Notes 16** and **17**).
8. Keep samples on ice until ready to be analyzed (*see* **Note 18**).
9. Proceed to analyze the nuclear DNA content, vortexing the sample before putting it on the flow cytometer (follow instructions in Subheading 3.2).

3.1.2 Isolation of Plant Nuclei Using the Two-Step Protocol

This procedure uses either the Otto or Baranyi buffers (buffer 11 or 12, respectively, listed in Subheading 2.3.2).

1. Place a small amount of the selected plant tissue (usually about 1 cm^2 or 20 mg) in a 6 cm petri dish (*see* **Note 11**).
2. Add 1 mL of ice-cold Otto I or ice-cold Baranyi solution I buffer (*see* **Note 12**).
3. Chop the tissues in the buffer using a new razor blade or sharp scalpel (*see* **Note 13**).
4. Mix the crude suspension by gently shaking the petri dish.
5. Filter the homogenate through a 30–42 μm nylon mesh filter into a labelled 1.5 mL tube.

6. Pellet the nuclei by centrifuging at 150 g for 5 min (*see* **Notes 19** and **20**).
7. Carefully remove the supernatant leaving approximately 100 μL of the buffer (*see* **Note 21**).
8. Resuspend the pellet by gently shaking and add a further 100 μL of the buffer used in **step 2** (*see* **Note 22**).
9. Add 1 mL of room temperature buffer, either Otto II or Baranyi solution II (*see* **Note 23**).
10. Add the appropriate volume of the fluorochrome to the nuclei suspension (if it is not already in the buffer—*see* Subheading 2.3.2) and vortex gently. For a typical sample which is c. 1.2 mL, the amount of stock PI added is 60 μL, while for DAPI, 50 μL should be added.
11. Incubate the samples at room temperature for few minutes in the dark (*see* **Note 24**).
12. Proceed to analyze the nuclear DNA content, vortexing the sample before putting it on the flow cytometer (follow instructions in Subheading 3.2).

3.1.3 Isolation of Plant Nuclei Using a Simplified Two-Step Protocol

This procedure uses either Otto, Baranyi, or Mishiba's buffer (buffers 11, 12, or 13, respectively, listed in Subheading 2.3.2).

1. Place a small amount of the selected plant tissue (usually about 1 cm^2 or 20 mg) in a 6 cm petri dish (*see* **Note 11**).
2. Either (1) add 0.5 mL of ice-cold Otto I or ice-cold Baranyi solution I buffer, or (2) add 0.2 mL of ice-cold Mishiba's solution A (*see* **Note 12**).
3. Chop the tissues in the buffer using a new razor blade or sharp scalpel (*see* **Note 13**).
4. Mix the crude suspension by gently shaking the petri dish. If using Mishiba's buffer, incubate for 5 min at room temperature.
5. Either (1) add 2 mL of Otto II or Baranyi solution II buffer, or (2) add 1 mL of Mishiba's solution B.
6. Filter the homogenate through a 30–42 μm nylon mesh filter into a labelled flow cytometry tube (*see* **Note 15**).
7. Add the appropriate volume of the fluorochrome to the nuclei suspension (if it is not already in the buffer—*see* Subheading 2.3.2) and vortex gently.
   (a) For a typical sample using either Otto or Baranyi buffers, the volume is usually c. 2.5 mL; thus, the amount of stock PI added is 125 μL, while for DAPI, 100 μL should be added.
   (b) For a typical sample using Mishiba's buffer, the volume is usually c. 1.2 mL; thus, the amount of stock PI added is 60 μL, while for DAPI, 50 μL should be added.

8. (a) For samples in the Otto or Baranyi buffer, incubate at room temperature for few minutes in the dark (*see* **Note 24**).
   (b) For samples in Mishiba's buffer, incubate at room temperature in the dark for 20 min.
9. Proceed to analyze the nuclear DNA content, vortexing the sample before putting it on the flow cytometer (follow instructions in Subheading 3.2).

3.2 Analysis of the Nuclear DNA Content and DNA Ploidy Level

The flow cytometer allows the measurement of several optical properties of the isolated particles (i.e., nuclei) that move one by one through the flow capillary tube illuminated by a laser beam or mercury light source. Prior to analyzing any plant sample, check that the instrument is properly aligned using fluorescent calibration beads (*see* Subheading 2.2). Subsequently test the linearity of the flow cytometer by running a plant sample (e.g., reference standard) and comparing the ratio between the $4C/2C$ peaks, which ideally should be in the range of 1.98–2.02 *sensu* Doležel et al. [8].

The first step in the analysis of a new target species requires the user to determine its relative nuclear DNA fluorescence. This step is described in Subheading 3.2.1. Based on this information, the user can then proceed either to Subheading 3.2.2 to determine the absolute DNA amount or to Subheading 3.2.3 to determine the DNA ploidy level.

3.2.1 Measurement of the Relative Nuclear DNA Fluorescence of a Sample

1. Load the tube containing the suspension of stained nuclei onto the flow cytometer sample port and run for a few seconds at low speed until the flow has stabilized through the tubing system (*see* **Notes 25** and **26**).
2. Adjust the flow rate to a speed of 15–25 nuclei/s (*see* **Notes 27** and **28**).
3. Once the sample is running through the flow cytometer, a flow histogram with peaks will start to appear. The peak positions can then be adjusted using the instrument gain settings to move the peaks within the histogram (*see* **Notes 29** and **30**). It is also possible to adjust the lower limit threshold so that undesirable low-channel signals (e.g., from cell debris and autofluorescent compounds) are excluded from the histogram. If there is a large amount of cell debris/background fluorescence in the flow histogram, then *see* **Note 31**, while if additional, unexpected peaks appear, then *see* **Note 32**.
4. Measure 5,000 particles (*see* **Note 33**).
5. Use the software provided by the flow cytometer manufacturer to assess the quality of histograms by (1) estimating the proportion of background, (2) checking peak symmetry, and (3) evaluating the peak width, expressed as the coefficient of variation, CV% (=SD of peak/mean channel position of the peak × 100) (*see* **Notes 34** and **35**).
6. Save the histogram if appropriate (*see* **Note 36**).

3.2.2 Measurement of the Absolute Nuclear DNA Content of a Sample Using a Reference Standard

Once the target sample has been run on its own to determine what reference standard to use and what gain the machine should be set at (*see* Subheading 3.2.1), a combined sample which includes both the target species and reference standard can then be prepared and run to determine the absolute nuclear DNA content of the target species.

To ensure the estimate of nuclear DNA content in absolute units is as accurate as possible, FCM researchers have adopted several best practice approaches. These include the following recommendations: (1) three specimen plants are collected per population/species and three independent replicates are processed per sample, or (2) five specimens are collected per population/species and two independent replicates are processed per specimen. (3) Only intercalating fluorochromes (e.g., PI) should be used; base-specific fluorochromes such as DAPI are not suitable for estimating nuclear DNA content.

1. Load the sample which contains a suspension of stained nuclei of both the target species and the selected internal reference standard (based on results obtained in Subheading 3.2.1) onto the flow cytometer sample port and run for a few seconds at low speed until the flow has stabilized through the tubing system (*see* **Notes 25** and **26**).
2. Adjust the flow rate to a speed of 15–25 nuclei/s (*see* **Notes 27** and **28**).
3. Once the sample is running through the flow cytometer, a flow histogram with peaks will start to appear. The peak positions can then be adjusted, if necessary, using the instrument gain settings to move the peaks within the histogram. It is also possible to adjust the lower limit threshold so that undesirable low-channel signals (e.g., from cell debris and autofluorescent compounds) are excluded from the histogram.
4. Check to see if there is any evidence of negative effects caused by the presence of cytosolic compounds which can affect the accuracy of the *C*-value estimation. This is done by comparing the position of the G_1 peak of the reference standard in this combined sample with its position in a sample containing just the reference standard (*see* Subheading 3.2.1) (N.B. both samples must be run at the same gain).
5. When this situation arises, alternative isolation methods should be tested (*see* **Note 37**); otherwise, proceed to the next step.
6. Measure 5,000 particles (*see* **Note 33**) (in some protocols, 10,000 particles are recommended) and save the data (*see* **Note 36**).
7. Use the software provided by the flow cytometer manufacturer to assess the quality of histograms (*see* **step 5** of Subheading 3.2.1). Assuming the quality of the histograms is suitable (i.e., CVs<3 %) (*see* **Note 34**), also obtain the statistical information for the histogram (i.e., mean peak position).

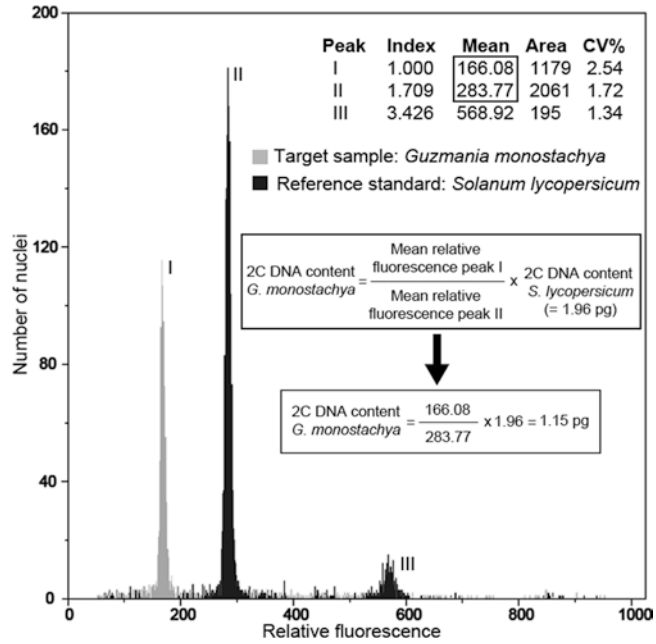

Fig. 3 A typical flow histogram to illustrate how genome size is calculated for the target species *Guzmania monostachia* using *Solanum lycopersicum* as the internal reference standard. Using the output data from the flow cytometer software, the mean relative fluorescence of the G_1 peak of *G. monostachia* (*grey peak* labelled I, i.e., 166.08) is divided by that of the mean G_1 peak of the standard *S. lycopersicum* (*black peak* labelled II, i.e., 283.77). This ratio is then multiplied by the 2C DNA content of *S. lycopersicum* to give the 2*C*-value of *G. monostachia*. To convert between pg and Mbp, use the conversion factor 1 pg = 978 Mbp Dolezel et al. [1]. (N.B. peak III is the G_2 peak of the reference standard.)

8. Calculate the nuclear DNA amount (2*C*-value) of the target plant in each replicate as follows (*see* **Notes 38** and **39**):

$$2C\text{ DNA content target (pg)} = \frac{\text{target sample mean } G_1 \text{ peak}}{\text{standard sample mean } G_1 \text{ peak}} \times 2C\text{ DNA content standard (pg)}$$

For an illustrative sample histogram output and calculation, *see* Fig. 3.

9. Calculate the mean nuclear DNA content and the standard deviation for the species (including all specimens and replicates) (*see* **Note 40**). (N.B. to convert between picograms (pg) and megabase pairs (Mbp) use: 1 pg = 978 Mbp [1]).

3.2.3 Measurement of the Relative Nuclear DNA Content of a Sample Using a Reference Standard to Determine DNA Ploidy Level

Among the multiple uses of FCM, DNA ploidy estimation is becoming highly popular as it allows the rapid screening of multiple samples. The protocol described below is optimized to work at either the species level or within species complexes.

1. Load the sample which contains a suspension of stained nuclei of both the target species of unknown ploidy and either a reference sample comprising a species of known ploidy (i.e., karyologically determined) or another internal standard (in that case, as mentioned above, the ploidy level of at least one target sample must be karyologically determined) (*see* **Note 41**) onto the flow cytometer sample port, and run for a few seconds at low speed until the flow has stabilized through the tubing system (*see* **Note 25**).
2. Perform **steps 2–3** (Subheading 3.2.2).
3. Measure at least 3,000 particles (*see* **Note 42**) and save the data (*see* **Note 36**).
4. Use the software provided by the flow cytometer manufacturer to obtain the statistical information for the histogram (e.g., peak position and ratio, area, CV%).
5. Calculate the relative nuclear DNA amount (DNA ploidy) of the target plant as follows:
   (a) If the reference sample used (with known ploidy) is the same species as the target sample, a perfect overlapping of G_1 peaks will indicate they both have the same ploidy.
   (b) If multiple peaks appear, then calculate the ploidy level using the following formula:

$$\text{Target sample ploidy} = \frac{\text{target sample mean } G_1 \text{ peak}}{\text{standard sample mean } G_1 \text{ peak}} \times \text{reference sample ploidy.}$$

   (c) If one of the cultivars listed in Table 1 is used as the reference standard, ploidy levels can be inferred by means of the ratio between the G_1 peaks of both the standard and the target samples (keeping in mind that the chromosome number of at least one of the target samples must be known). An example of the FCM analysis of ploidy level in the genus *Sorbus* (Rosaceae) is given in Fig. 4.

4 Notes

1. Many of the chemicals that are used in FCM are considered hazardous, and so suitable protective equipment (i.e., gloves, lab coat, fume cupboard) should be used to avoid health risks, and manufacturer's safety recommendations should be followed when using them. For example:
   MOPS (3-morpholino-propanesulfonique acid) and DTT (dithiothreitol) may cause irritation to the eyes, respiratory system, and skin.

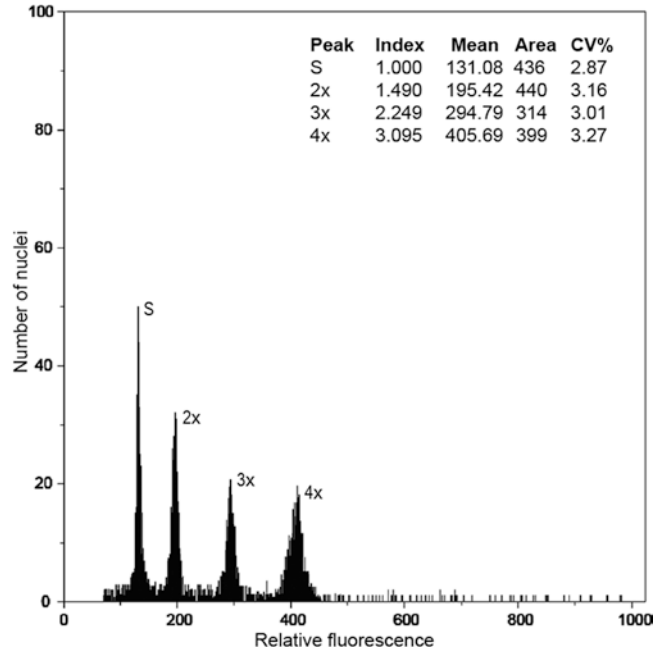

Fig. 4 Flow cytometric ploidy analysis in *Sorbus*. DNA ploidy was assessed in different species of *Sorbus* using the internal reference standard (*Oryza sativa*). Diploid *Sorbus aria* (whose chromosome number has been counted) was used as a reference to uncover higher ploidy levels in related species of unknown ploidy by determining the ratio between the peaks of these *Sorbus* species and the internal reference standard. S = G_1 peak of the internal standard (*Oryza sativa*); 2x = G_1 peak of the chromosomally determined diploid *S. aria*; 3x = G_1 peak of the triploid *S. saxicola*; 4x = G_1 peak of the tetraploid *S. rupicola*

β-mercaptoethanol is very hazardous and can be fatal if inhaled, swallowed, or absorbed through skin contact.

PI is a potential mutagen and may cause irritation to the eyes, respiratory system, and skin.

DAPI is a potential carcinogen and may cause irritation to the eyes, respiratory system, and skin.

DMSO (dimethyl sulfoxide) itself is not considered as a hazardous substance but in contact with other potentially toxic chemicals might enhance their absorption through the skin.

2. PI and SYBR Green I are intercalating fluorescent dyes that bind to double-stranded DNA and RNA with no base preference, whereas DAPI is a fluorescent dye that binds preferentially to AT-rich DNA.
3. Some buffers might precipitate after a while if they have not been stored at the appropriate temperature or when poor quality water has been used. It is therefore recommended that buffers are stored at the specified temperature given in

Subheading 2.3.2 and, wherever possible, that high-quality single- or double-distilled water is used.

4. If the isolation buffer becomes cloudy, changes color, or contains suspended particles, it suggests that the buffer has been stored incorrectly or that the storage time has been exceeded. In either case, this can result in fungi or bacteria growing in the buffer. If this has happened, then new isolation buffer needs to be prepared and stored as indicated (*see* Subheading 2.3.2). Unused buffer should be discarded after 3 months. It is also strongly recommended to prepare small volumes (e.g., 200 mL) so that the stocks are as fresh as possible.
5. The pH of the isolation buffers must be above 4 in order for PI to stain the DNA; most are around a neutral pH. For protocols using either Otto buffer (*see* buffer 11, Subheading 2.3.2) or Baranyi buffer (*see* buffer 12, Subheading 2.3.2), the nuclei are isolated in a citric acid solution which is acidic (i.e., Otto I or Baranyi solution I). The pH is then raised to neutral by the addition of a basic solution containing Na_2HPO_4 (i.e., Otto II or Baranyi solution II) to ensure optimum staining of the DNA when the fluorochrome is added.
6. Isolation buffers contain a number of different components which ensure that not only are sufficient numbers of nuclei released from the cytoplasm but also that the DNA is protected from degradation and binds the fluorochrome quantitatively. Typically isolation buffers include the following components: (1) organic buffers (e.g., Tris, MOPS, HEPES) which stabilize the pH between 7.0 and 8.0 (depending on the buffer) to enable DNA staining by the fluorochrome; (2) nonionic detergents (e.g., Triton X-100 and Tween 20) to facilitate the release of nuclei and prevent their aggregation; (3) chromatin stabilizers (e.g., spermine, $MgCl_2$, $MgSO_4$) to maintain the integrity of the DNA; (4) chelating agents (e.g., Na_2EDTA (ethylenediaminetetraacetic acid disodium salt), sodium citrate) to bind divalent cations such as Mg^{2+} and Mn^{2+} and hence block DNase activity; and (5) inorganic salts (e.g., KCl, NaCl) to ensure the correct ionic strength of the buffer. Some buffers also include β-mercaptoethanol, DTT, ascorbic acid, and sulphite which act as reducing agents to prevent protein oxidation and PVP (*see* **Note 7** below). For a discussion of the effect of different buffer components in a range of plant species, *see* Loureiro et al. [45] and Greilhuber et al. [46].
7. The polymer PVP is used to reduce the effect of polyphenols and other secondary metabolites such as tannins that are often present in plant tissues and which can inhibit the quantitative staining of DNA by the fluorochrome. Such secondary metabolites may also increase cell debris leading to a significant reduction in the quality of the peaks in the flow histogram (*see* **Notes 31** and **35**). Generally PVP-10 and PVP-40 are used

although in certain cases only PVP-360 was shown to result in decent flow histograms (*see* Fig. 5a).

8. A modified version of this buffer was reported by E Hörandl et al. [47] who also added 6.1 mM sodium citrate to the buffer.
9. It is *essential* that the cell culture-tested grade of Tween 20 from Sigma-Aldrich (cat. no. P2287) is used. Tween 20 for molecular biology (Sigma cat. no. P9416) is not suitable for FCM.
10. Dissolving $Na_2HPO_4.12H_2O$ can be speeded up by heating the solution gently.
11. The amount of tissue used needs to be determined empirically, taking into account the amount of nuclei released and the proportion of debris produced. For internal standardization, when needed, also add leaf tissue of the appropriate reference standard species (*see* Table 1).
12. The selection of the most appropriate buffer needs to be determined empirically for each plant group. In many cases, the same buffer works well across a family, while in other cases, different buffers are needed for different genera, or even within a genus.
13. It is very important to use very sharp razor blades or scalpels to chop the tissue into a crude suspension while minimizing damage to the nuclei. It is therefore recommended that each razor blade or scalpel is used only once. The chopping must be vigorous, quick, and short to avoid drying of the sample. We recommend empirical adjustments, especially to the chopping intensity, so that optimal numbers of nuclei are released without generating too much cell debris which can lead to high background signal in the flow histogram and low numbers of nuclei in the G_1 peak.
14. The working volume can be modified, but remember that if this is done, then the volume of the fluorochrome added at **step 7** will need to be adjusted accordingly to maintain the appropriate final concentrations.
15. Check carefully that the sample is free of particles after filtration to minimize the possibility of blockages in the flow cytometer.
16. If the samples have become brown/dark just a few minutes after adding the fluorochrome, this is indicative that the sample is undergoing oxidation due to the negative effect of secondary metabolites present in the cytoplasm. To avoid this problem, it is recommended that the buffer is supplemented with reducing agents such as β-mercaptoethanol or DTT. Another option that might help is the addition of PVP-10, PVP-40, or higher molecular weights such as PVP-360 (*see* **Note 7**), which will help improve histogram quality and sample stability, especially

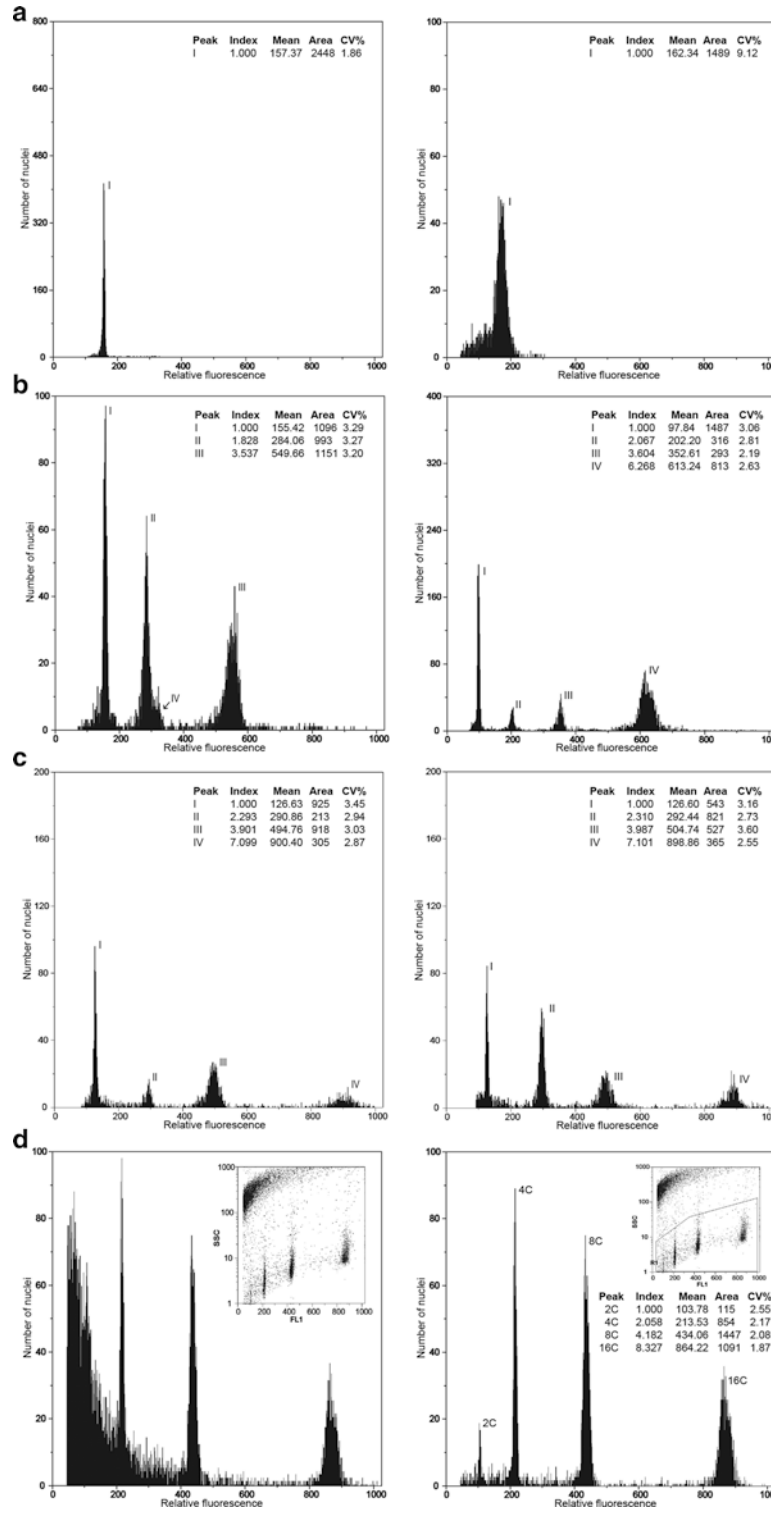

Fig. 5 Troubleshooting problems encountered during flow cytometric analysis of plant material. (**a**) Fluorescence histograms obtained after analysis of isolated nuclei of *Clusia multiflora* (Clusiaceae). Samples in both histograms were

if tannins are present. If the problem persists, then alternative isolation buffers (*see* Subheading 2.3.2) should be tested and the chopping intensity reduced (*see* **Note 13**).

17. Many protocols add RNase (ribonuclease II-A) at 50 μg/mL at this stage when PI is used as the fluorochrome. This is because PI intercalates into double-stranded (ds) nucleic acids so they can stain dsRNA as well as dsDNA. Nevertheless, since RNase is only active between 15 and 70 °C, with an optimal temperature of 60 °C, it can be left out of any protocol that lacks an incubation step within this temperature range. Since the protocols described here do not include such an incubation step, RNase has not been included. Nevertheless, if users want to include an RNase incubation step, a stock RNase solution can be prepared by heating 1 mg/mL RNase to 80 °C for 15 min (to inactivate DNases) and filtering through a 0.22 μm filter. The stock can be stored in 1 mL aliquots at −20 °C.
18. The time between staining (**step 7**) and running the sample on a flow cytometer can vary from a few minutes to up to 1 h. While for some plant samples, a short incubation works fine, for others a longer incubation can give better results. Thus, the incubation time needs to be adjusted empirically for a given plant species to optimize the results.
19. The relative centrifugal speed and time may need to be empirically adjusted.

Fig. 5 (continued) prepared using the same leaf and the same isolation buffer (WPB—36) but supplemented with different types of polyvinylpyrrolidone (PVP) to illustrate the dramatic effect on the quality of the flow histogram (*left*) using PVP-360 and (*right*) using PVP-40. (**b**) Flow histograms of the relative fluorescence in *Dactylorhiza* sp. (Orchidaceae) illustrating the utility of using alternative tissues to leaf samples to estimate nuclear DNA contents. (*Left*) Genome size estimated using pollinia of *Dactylorhiza* sp. and *Solanum lycopersicum* as internal standard [standard: peak I (G_1) and IV (G_2); pollinia: peaks II ($1C-G_1$) and III ($2C-G_2$); calculated 1*C*-value of *Dactylorhiza* sp. = 3.58 pg]. (*Right*) Genome size estimated using leaf tissue of *Dactylorhiza* sp. and *Solanum lycopersicum* as internal standard [standard: peak I (G_1) and II (G_2); *Dactylorhiza* sp. leaf: peaks III ($2C-G_1$) and IV ($4C-G_2$); calculated $2C = 7.06$ pg]. (**c**) Flow histograms of leaf tissue from the orchid *Dracula* sp. (using *Oryza sativa* as internal standard (peak I)), illustrating how different parts of the same leaf can have very different proportions of G_1 and G_2 nuclei. Using a young and actively growing leaf of *Dracula* (c. 1.5 cm long), the apical tip was seen to have a much lower proportion of G_1 nuclei (peak II, left histogram) compared with the basal part of the leaf (peak II, right histogram). (N.B. peaks III and IV correspond to G_2 and partial endopolyploid nuclei, respectively). (**d**) Flow histograms of relative fluorescence in leaf tissue of *Kalanchoe marnieriana* (Crassulaceae) illustrating how poor histograms with much debris (*left*, ungated histogram) can be improved by gating the histogram (*right*) to reveal not only the G_1 nuclei of *K. marnieriana* which was hidden in the debris of the left histogram but also the presence several endopolyploid cycles

20. Samples are stable in Otto I and Baranyi solution I; hence, it is possible to prepare several samples in advance and simultaneously centrifuge them together.
21. It is important to do this step very gently so as not to remove the pelleted nuclei.
22. As samples are stable in Otto I and Baranyi solution I, it is possible to prepare many replicates and store them at either room temperature or 4 °C for up to several hours.
23. The addition of Otto II or Baranyi solution II raises the pH of the sample to c. 7.3 and increases salt concentration. To keep these parameters within a working range, the amount of buffer added at this stage should be about fourfold that of Otto I or Baranyi solution I which now comprises c. 200–250 μL.
24. The optimal incubation time should be adjusted in each case, but short incubation times (e.g., less than 5 min) usually provide the best results because nuclei may not remain stable for a long time after this step.
25. The user can clear the acquisition results as many times as needed until the flow rate becomes stabilized. Do not be tempted to start recording data for analysis until the flow rate has stabilized (usually 0.5–1 min after the start of the run), as this can lead to poor histograms and inaccurate results.
26. If no peaks are appearing in the flow histogram, and assuming that the flow cytometer is properly set up, the peaks are probably off the scale due to an inappropriate gain setting for the sample being analyzed. To locate the peaks, adjust the gain setting of the machine. This can sometimes be done more easily by using the log scale setting of the relative fluorescence (x axis) scale. Once the position of the peak has been located, adjust back to a linear scale to perform analyses. Remember that the gain of the machine should be kept within the range that is recommended by the manufacturer of the flow cytometer to ensure the machine is operating optimally.
27. If the flow rate is slow, there are a number of explanations and possible solutions: (1) this could be a technical problem with the flow cytometer. Any blockage in the flow chamber or in the tubing system can cause a reduction in the number of nuclei recorded. Check that the pressure in the system is within the recommended range for the machine and clean the flow system using either a decontaminant solution or a diluted bleach solution (*see* Subheading 2.2) to wash out any potential blockage. (2) Alternatively, this could be a biological problem caused by the particular plant material being analyzed. The concentration of nuclei in the suspension can vary significantly between samples depending on tissue type, quantity of material used, etc. Hence, the flow rate will need to be adjusted each time a new sample is loaded onto the machine. If the concentration of

nuclei in the sample is low, this will necessitate a high flow rate, and this can result in a broadening of peaks and high CVs (*see* **Notes 34** and **35**). If possible, it is best that this is overcome by preparing a new sample using more material to increase nuclei concentration, rather than running the sample at a high flow rate. When using flow cytometers with a preset sample acquisition rate (e.g., slow, medium, high), we recommend using the slow rate and only increase it if necessary.

Other possible causes of a slow flow rate include the following: inappropriate chopping intensity, the tissue used is not suitable, and/or the isolation buffer selected is not appropriate. Such problems can be overcome by, for example, increasing the amount of tissue used, adjusting the chopping intensity, and testing different types of plant material (*see* Subheading 2.1, and Fig. 5b which illustrates the effect of changing from leaf material to pollinia in the orchid *Dactylorhiza*). Even changing the end of the leaf used for analysis can result in a dramatic change in the proportion of G_1 nuclei released (*see* Fig. 5c). Changing the isolation buffers (*see* Subheading 2.3.2) can also have a large effect, especially if the sample is releasing mucilaginous compounds into the chopping buffer. Indeed, many plants contain mucilage in their cytoplasm, and isolated nuclei may bind to this during the chopping process leading to a low number of released nuclei. Increasing the percentage of detergent (i.e., Triton X-100 up to 4 %) can help but keep in mind that a higher concentration of detergent can also result in higher levels of cell debris and hence poorer quality of flow histograms, so compromise may be necessary.

28. If the flow rate is unstable just after starting acquisition and large numbers of particles are being recorded, even when the flow cytometer is running at a slow speed, this may be due to unstable pressure in the flow cytometer. It can be caused by a number of factors including the presence of suspended particles (e.g., algae) in the sheath fluid and sheath fluid tubes/filters. Check that the pressure is correct and replace sheath fluid, tubes, and filters. If algae become a recurrent problem, 0.02 % sodium azide can be added to the water in the sheath fluid bottle; however, it should be noted that sodium azide is toxic and should be handled appropriately. Alternatively, the sheath fluid bottles can be thoroughly rinsed with domestic bleach (*see* Subheading 2.2) every 2 months, or even more frequently when they are not changed on a daily basis. In addition, many manufacturers recommend that the sheath fluid tubing and filters are replaced every 3 months.

29. Given that most of the measurements will require the use of a reference standard (*see* Subheadings 3.2.2 and 3.2.3), it is strongly recommended that the user knows, in advance, the peak position of a set of reference standards, ranging from small to big genomes (check Table 1 for recommended reference standards). This can be done by adjusting the gain set-

tings so that the G_1 peak of the standard always falls, for example, around channel number 200. Then, when the target sample is run alone for the first time, the user will be able to determine the best reference standard by testing the peak position of the target plant at the different gains selected for the standards. It should be noted that while the G_1 peak is the dominant peak, in many cases, G_2 peaks are present which might interfere with the target sample. Care should therefore be taken to note where the peak positions of the target sample fall in relation to both the G_1 and G_2 peaks of the reference.

The position of the G_1 peaks in the flow histogram of both the target plant and the internal reference standard should be different enough to avoid overlapping peaks. However, ideally, the ratio between the standard and the target plant peaks should not exceed threefold to reduce risk of errors arising due to loss of linearity.

30. If the position of the peak appears to be unstable (i.e., the peak in the histogram builds at a different position each time the acquisition data is cleared), it may suggest the incubation time following the addition of the fluorochrome is insufficient (i.e., **step 8**, Subheading 3.1.1). Check different incubation times to test staining stability. If the problem persists, test alternative isolation buffers. However, when using Otto or Baranyi buffers, the nuclei may be unstable once Otto II or Baranyi solution II has been added (*see* **step 11**, Subheading 3.1.2 or **step 8**, Subheading 3.1.3). For these buffers, increasing the incubation time is only likely to lead to deterioration in the flow histogram quality and unstable peaks.

31. Large amounts of cell debris/background on the flow histogram are a commonly encountered problem (e.g., see histogram on the left of Fig. 5d). There are several explanations and solutions:

    (a) The isolation buffer selected is not appropriate for the sample. Test an alternative isolation buffer (*see* Subheading 2.3.2).

    (b) The tissue selected is not in good condition or optimal for FCM. Test other plant tissues (*see* Subheading 2.1).

    (c) The incubation time (**step 8**, Subheading 3.1.1, **step 11**, Subheading 3.1.2, **step 8**, Subheading 3.1.3) can influence the quality of the flow histogram so try adjusting the incubation time.

    (d) Over-chopping of the sample (*see* **step 3** in Subheadings 3.1.1, 3.1.2, and 3.1.3) can, in some cases, lead to large amounts of background debris in the flow histogram. Reduce chopping intensity and use new sharp razor blades or scalpels for each sample to avoid cell damage.

    (e) If none of the solutions mentioned so far are showing any improvement in the results, then in some cases, gating can be used if the flow cytometer is fitted with a side

scatter detector. In this case, the region of interest is selected in the side scatter vs. forward light histogram so that the flow histogram of relative fluorescence excludes the signals from side scatter. An example of how effective this can be is seen in Fig. 5d.

32. If additional and perhaps unexpected peaks which do not follow an endopolyploid series are present in the flow histogram, then this suggests the presence of contaminants such as insects, insect eggs, and fungi in the plant sample. To avoid this problem, always check the plant material carefully before chopping (using a stereomicroscope if necessary) to ensure there are no contaminating organisms. If endoparasites are suspected, then alternative plant parts will need to be tested.
33. The number of particles that need to be recorded will vary depending on the type of analysis being carried out. Usually 5,000 particles are recorded of estimations of genome size, and this is the recommended number, but for some material, it may not be possible to obtain so many nuclei (e.g., recalcitrant and herbarium material).
34. The CV of a peak is a measure of peak quality and must be kept as low as possible (ideally less than 3 %) and always below 5 %. Higher CVs are not acceptable for publication unless it has been demonstrated that higher quality cannot be achieved after extensive tests with different buffers, incubation times, etc. (e.g., samples rich in polyphenols, old silica-dried samples, and herbarium vouchers).
35. Broad peaks with high and unacceptable CVs are, unfortunately, commonly encountered in the analysis of plant material. There are several possible explanations and solutions. These can broadly be divided into technical and biological sources:

    Technical

    (a) A loss of pressure in the flow cytometer system might result in a reduction of the peak quality. Check that the pressure is correct.

    (b) The instrument might be out of alignment. Align the instrument light source by using calibration beads (*see* Subheading 2.2).

    (c) Broad peaks are produced when the flow rate is too high. Run the samples at a flow rate that is no greater than c. 20 nuclei/s.

    (d) Air bubbles in the flow system can cause peaks with high CVs. Clean the flow chamber as recommended by the manufacturer and take extra care to remove any air bubbles from the filter after the sheath fluid bottle has been refilled. Also make sure that the lid of the sheath fluid bottle is tightly screwed on to seal the system.

(e) As reported by Doležel et al. [8], an obsolete arc lamp used for UV excitation might be a cause for such problem. Replace the lamp and align the instrument.

(f) Weak fluorescence and peaks with large CVs can arise when a sample of DAPI-stained nuclei is analyzed following a sample of PI-stained nuclei. Doležel et al. [8] noted that this situation can arise as a result of fluorochrome interference if the flow cytometer has not been completely cleaned between samples. To avoid this problem, ensure that the machine is thoroughly washed through by running a tube containing a weak solution (1:5 dilution in distilled water) of domestic bleach (do not leave bleach sitting in the system for more than a few minutes), then washing the system thoroughly with distilled water.

Biological

(a) In some cases, the isolation protocol and/or the buffer used is unsuitable for the material being analyzed, and the result can be a poor quality flow histogram. Test alternative isolation buffers (Subheading 2.3.2) and protocols (Subheading 3.1).

(b) Secondary metabolites in the cytoplasm may interfere with the fluorochrome staining of the DNA and lead to an increase in the CVs. Sometimes this can be overcome by supplementing the buffer with reducing agents such as β-mercaptoethanol or DTT. Tannins are also frequent in plants, so the addition of PVP-10/PVP-40 is common to help minimize their effects. PVP-360 has also been shown to be effective and, in certain cases, may work when other PVP types have failed (e.g., *see* Fig. 5a). The effect of secondary metabolites can also be minimized by reducing the chopping intensity and carrying out the nuclei isolation steps on ice and with ice-cold solutions (as recommended in Subheading 3.1).

(c) Some tissues of some plants are just recalcitrant and produce poor results. Test alternative tissues, such as bracts, roots, sepals, seeds, and pollinia (e.g., *see* Fig. 5b) of different parts of the leaf (e.g., *see* Fig. 5c) or try putting the plant in the dark for a few days prior to analysis.

(d) Doležel et al. [8] reported that excluding RNase from the isolation buffer when PI is used to stain DNA can result in increased CVs, especially in tissues with active protein synthesis, such as root tips. Nevertheless, if this is the case, then the protocol should include an incubation step at 37 °C for at least 30 min to ensure the RNase has sufficient time to work (*see* **Note 17** for how to prepare RNase).

(e) The wrong concentration of the DNA fluorochrome can also reduce the quality of the flow histogram, so it is important to check that the fluorochrome solution has been prepared correctly.

36. It is recommended that when a run is saved, the file name should include information on the species analyzed, replicate number, buffer used, and internal reference standard (if applicable). If possible, it is also helpful to get the software to list the instrument settings (e.g., gain and lower limit settings) used for each run. This enables histograms to be compared, if appropriate.
37. If a shift in the position of the G_1 peak of the reference standard is detected, then it is necessary to change the sample preparation. Often, the problem can be solved by changing to another isolation buffer (*see* Subheading 2.3.2). Alternatively, the addition of various compounds can sometimes eliminate the problem, e.g., the addition of 3 % PVP (*see* **Note 7** and Fig. 5a) to bind to polyphenolics or adding DTT and β-mercaptoethanol which are good reducing agents. In addition, the problem can sometimes be overcome by using different plant material such as roots, stems, bracts, or seeds (e.g., *see* Fig. 5b, c).
38. For accurate nuclear DNA amount estimations, it is recommended that the number of particles in both the target and the reference standard G_1 peaks should be similar.
39. Some plant breeding material, pollen, and the gametophyte stage of bryophyte groups (i.e., mosses, liverworts, and hornworts) are haploid. In such cases, the first peak of the target sample in a flow histogram (G_1) will correspond to the 1*C* rather than the 2*C*-value.
40. Technical factors should not account for more than 2–3 % of the variation between different estimates for the same species, although for some material (e.g., recalcitrant tissues), this type of variation may be greater. Higher levels of variability in *C*-value estimates for a species may reflect intraspecific variation due to the presence of chromosomal instabilities (e.g., B chromosomes, supernumerary segments, or aneuploidy) or taxonomic heterogeneity in the samples analyzed (*see* ref. 48 for further discussion on intraspecific variation).
41. (1) Wherever possible, it is recommended that the "reference" sample of known ploidy is at the lowest ploidy level known for a given species/complex (i.e., diploids). (2) If investigating ploidy levels within a species, the reference sample can be a sample of this species whose ploidy has been karyologically determined (e.g., a diploid sample). (3) Following the recommendations of Doležel et al. [8], the nuclear DNA may be stained with PI or DAPI, although the latter option may result in higher-quality histograms. The use of DAPI is also recommended to detect aneuploid specimens.
42. For ploidy level estimations, it is not necessary to measure as many nuclei, so data for a lower number of particles can be collected.

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