Received: 6 February 2021 | Revised: 30 June 2021 | Accepted: 1 July 2021

DOI: 10.1002/cyto.a.24482

REVIEW ARTICLE

MIFlowCyt

ISAC CYTOMETRY Journal of Quantitative Cell Science PART A

Plant material selection, collection, preservation, and storage for nuclear DNA content estimation

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Funding information
Akademie Věd České Republiky, Grant/Award Number: RVO 67985939; CENTRO2020, Grant/Award Numbers: CENTRO-01-0145-FEDER-000007, CENTRO-01-0145-FEDER-000020; Fundação para a Ciência e a Tecnologia, Grant/Award Number: UIDB/04004/2020; Grantová Agentura České Republiky, Grant/Award Number: 20-22783S; Univerzita Karlova v Praze, Grant/Award Number: UNCE 204069

Abstract

In theory, any plant tissue providing intact nuclei in sufficient quantity is suitable for nuclear DNA content estimation using flow cytometry (FCM). While this certainly opens a wide variety of possible applications of FCM, especially when compared to classical karyological techniques restricted to tissues with active cell division, tissue selection and quality may directly affect the precision (and sometimes even reliability) of FCM measurements. It is usually convenient to first consider the goals of the study to either aim for the highest possible accuracy of estimates (e.g., for inferring genome size, detecting homoploid intraspecific genome size variation, aneuploidy, among others), or to decide that histograms of reasonable resolution provide sufficient information (e.g., ploidy level screening within a single model species). Here, a set of best practices guidelines for selecting the optimal plant tissue for FCM analysis, sampling of material, and material preservation and storage are provided. In addition, factors potentially compromising the quality of FCM estimates of nuclear DNA content and data interpretation are discussed.

KEYWORDS
Best practices, flow cytometry, plant sciences, nuclear DNA content, nuclear suspensions, plant material collection, plant material preservation, plant material storage, tissue selection

1 | TISSUE SELECTION

Generally, fresh and not fully-expanded leaves are recommended as plant material for DNA content estimation using FCM [1]. Very young, underdeveloped leaves (or immature organs in general) may exhibit distinct mitotic activity, whereas the use of senescing tissues may carry an increased risk of pathogen infestation or plant-controlled tissue degradation. Mature and older plant organs may also contain higher content of secondary metabolites interfering with FCM measurement (DNA staining in particular), and in some plant species such tissue is also prone to high levels of endopolyploidy. The importance of these adverse factors often varies across organs and tissue types, so whenever the results are unsatisfactory, the use of alternative plant material should be considered. For example, FCM analysis of petals gives better results than leaves for many Brassicaceae species, both with respect to quality (lower amounts of interfering metabolites and thus clearly delimited peaks) and interpretation (low endopolyploidy in petals; e.g., [2]). Pilot experiments to analyze materials from different organs and tissues (possibly also at different stages of their development) of the plants under study may be helpful when aiming for high-precision FCM measurements. Ideally, any sampled tissue should be intact, parasite- and pathogen-free and, whenever possible, the plants that are compared should be grown under identical environmental conditions, especially as related to light regime, which has

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been previously documented to affect the quality of FCM analysis via differential production of secondary metabolites [3]. For this reason, in case of ex situ cultivation the environmental conditions under which plant materials for FCM analysis have been grown (e.g., light and irrigation regime, soil quality) should be accurately reported and in detail.

1.1 | Mitotic activity and endopolyploidy

When employing FCM for quantification of nuclear DNA amount, usually irrespective of a particular application (e.g., genome size assessment, ploidy level determination), it is important to correctly assign the fluorescence intensity peak in the FCM histogram that represents nuclei with either somatic (2C) or gametic (1C) DNA content. While this is relatively straightforward in most cases, the identity of these desired peaks may be obscured if the analyzed material exhibits multiple peaks due to a high degree of cell division or endopolyploidy (Figure 1).

If an actively growing tissue is used for FCM analysis, a considerable portion of cells may have doubled their DNA content but remain in the G_2 phase of the cell cycle before entering mitosis and cell division. In this situation, the resulting FCM histogram typically consists of two peaks, one representing the desired G_0/G_1 phase nuclei and the other (with twice the fluorescence intensity and usually a substantially lower particle count—peak height) representing G_2 phase nuclei. Aside from that, the occurrence of more background noise between the two peaks and skewed bases of G_0/G_1 peaks toward higher DNA content may be observed as a result of the presence of S phase nuclei at various stages of DNA synthesis. Problems usually occur when the G_0/G_1 phase nuclei peak is hidden among fluorescent debris or overlaps with the peak of the internal standard. To minimize the proportions of G_2 and S phase nuclei, tissue selection should avoid root and shoot apical meristems, and plant organs at early developmental stages (e.g., very young, underdeveloped leaves).

Even more problematic can be the tissue of endopolyploid plants. Aside from somatic cells with the base DNA content of 2C (representing G_0/G_1 phase nuclei), a specific portion of cells in endopolyploid tissues undergoes one (4C), two (8C), three (16C), or even more rounds of genome duplication (endoreduplication) as part of their cell differentiation pathways (Figure 1D). The result is a series of nuclear fractions with fluorescence intensity consistently increasing by a factor of two (or exceptionally less—in some orchid species with "partial endoreduplication" [4, 5]), typically introducing several prominent peaks to a FCM histogram. A major pitfall is that the desired 2C nuclei often make up a small fraction of all nuclei in endopolyploid somatic tissues or may be even absent [6].

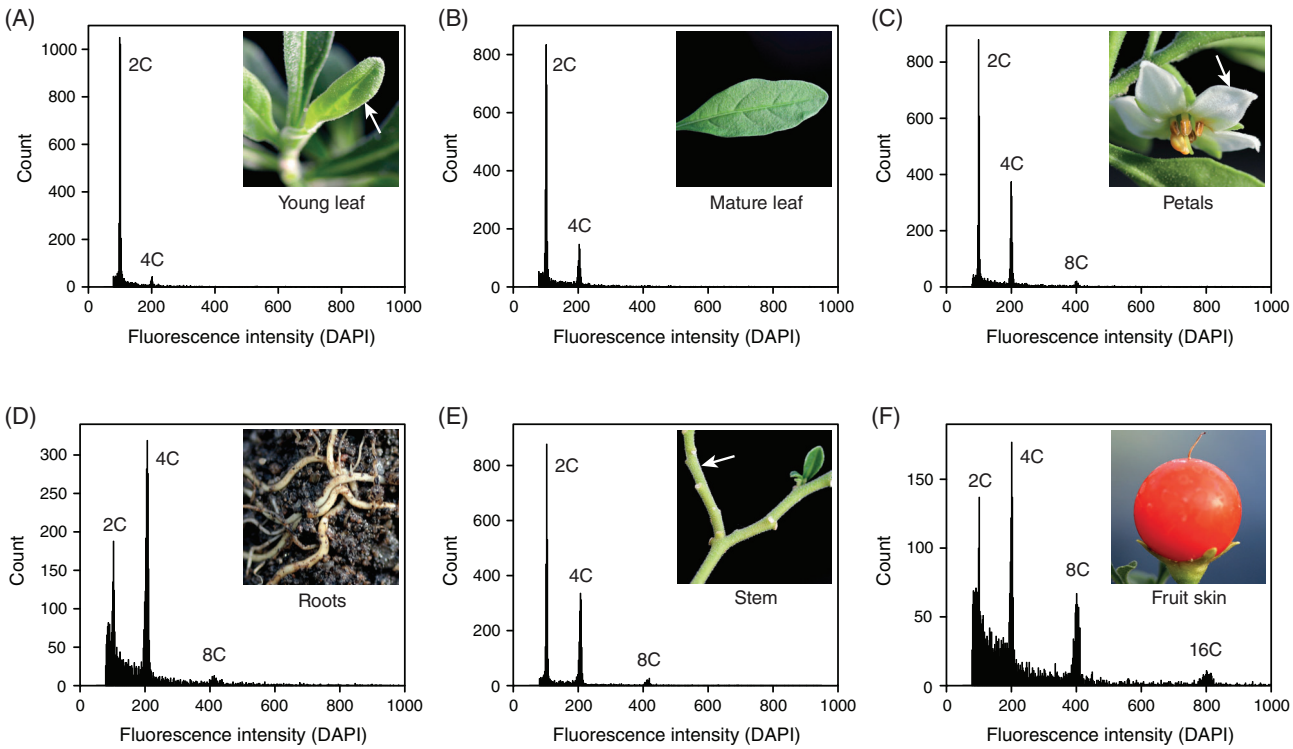

FIGURE 1 Alternative organ/tissue selection in Jerusalem cherry (*Solanum pseudocapsicum*), the resulting FCM histograms, and possible biases to nuclear DNA content estimation. (A) FCM analysis of a young leaf, the recommended first-choice option, rare 4C events represent either initiation of the endoreduplication process or actively growing cells in G_2 phase of the cell cycle. (B) A fully-developed leaf with a higher proportion of 4C events due to progressing endoreduplication. (C–E) Other plant organs exhibiting higher levels of endopolyploidy. (F) Highly endopolyploid fruit skin, where nuclei after 1–3 rounds of endoreduplication (4C, 8C, 16C) make up the majority of measured particles. Note the lower quality of analysis in (D,F), high background noise is due to increased intracellular content of interfering secondary metabolites [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 An overview of selected angiosperm plant families known to frequently impose challenges to FCM analysis along with the putative problems involved

Plant family	Problems
Aizoaceae	High endopolyploidy, organic acids (CAM), mucilage (succulency)
Amaranthaceae s.l.	High endopolyploidy
Anacardiaceae	Tannins
Boraginaceae	Interfering secondary metabolites (?)
Brassicaceae	High endopolyploidy
Cactaceae	High endopolyploidy, organic acids (CAM), mucilage (succulency)
Crassulaceae	High endopolyploidy, organic acids (CAM), mucilage (succulency)
Cucurbitaceae	High endopolyploidy
Ericaceae	Tannins
Euphorbiaceae	Milky sap (latex)
Fagaceae	Tannins
Geraniaceae	Tannins
Linaceae	Mucilage
Malvaceae s.l.	Mucilage
Myrtaceae	Tannins
Orchidaceae	High endopolyploidy (partial endoreduplication)
Oxalidaceae	Organic acids
Papaveraceae	Milky sap (latex)
Proteaceae	Tannins
Rosaceae	Tannins
Ulmaceae	Mucilage
Violaceae	Mucilage

The selection of species for study (whenever applicable) can be crucial, since the incidence of endopolyploidy has a strong phylogenetic component [7]. Some flowering plant families show a high degree of endopolyploidy across different species (Table 1); and both within and across plant families, endopolyploid species are more commonly found among short-lived herbs (annuals, biennials), succulents, and species with smaller genome size [8, 9]. Alternatively, these problems may be reduced during sample tissue selection. Even within individual, endopolyploidy is usually expressed to different degrees in particular organs and tissues ([10], Figure 1), and this is apparent from the earliest developmental stages (germinating seeds, seedlings; [11]). In general, the highest degree of endopolyploidy can be expected in endosperm, cotyledons (especially if these serve as a storage tissue, e.g., in Fabaceae), stamens (tapetum), and leaf stalks, whereas the opposite is true for gynoecia, leaf blades (except midrib and major veins) and particularly petals [8, 11, 12]. Trávníček et al. [4] pointed out that the only tissue consistently providing reliable genome size estimates across various orchid species was derived from young ovaries (2C nuclei only, meiosis is initiated after pollination) and pollinia (1C vegetative nuclei in pollen). In a similar manner, pollen or spores could be used for "calibration" of FCM histograms in other highly endopolyploid vascular plant groups, and sporophyte tissue (e.g., young capsules) might provide the same calibration for endopolyploid mosses, as suggested by Kron [6]. Additionally, it may be more convenient to work with immature plant organs that have not reached final levels of endopolyploidy (Figure 1A,B), and endopolyploid trichomes covering plant surface can be shaved off with a razor blade prior to tissue homogenization. It should be also noted, that the degree of endopolyploidy in a plant tissue may be also affected by environmental factors [9, 12, 13]. Pilot FCM analysis of various plant organs and tissues (potentially at different stages of their development) is thus highly recommended.

1.2 | Secondary metabolites interfering with FCM measurements

Diverse production of secondary metabolites is typical for plant cells and some of these cytosolic compounds have been shown to interfere with FCM analysis (e.g., by quenching the fluorescence of fluorochrome and/or binding to DNA molecules; for more details see Loureiro et al. [14]). Even though their chemical identities are poorly explored, in many cases these are phenolic compounds, such as anthocyanins, other flavonoids, and tannins [15]. High content of phenolics in a sample can be recognized based on the following [1, 3, 15]: (a) nuclear suspensions turning brown, (b) precipitation occurring in the nuclear suspensions, (c) markedly low quality of measurements (i.e., histograms with high levels of debris, broad peaks; Figure 1D,F), and (d) the fluorescence intensity of reference standard nuclei (i.e., the position of a respective peak on the x-axis of a FCM histogram) changing substantially depending on whether these were prepared alone or co-processed with sample nuclei. Moreover, the latter two indications should have general applicability across various interfering compounds.

Anthocyanins and other flavonoids, important for the coloration of flowers and fruits, are widely distributed across the plant kingdom. Since other compounds show degrees of taxonomic specificity, representatives of several plant families are recognized as posing serious challenges to FCM-based investigations (summarized in Table 1) and this should be taken into account when selecting a model species. High content of tannins is typical for some ferns and gymnosperms, but also for several angiosperm plant families (Table 1; [16]), and their adverse effect on FCM analysis has been nicely demonstrated by Loureiro et al. [17]. Tissues of some plant groups contain mucilaginous compounds that may complicate the filtering step in nuclei isolation protocols, or may even block the flow chamber of the cytometer. Other plant groups have tissues rich in organic acids (e.g., succulent families with CAM) and their analysis may disrupt the pH-buffering capacity of the isolation buffer.

Adverse effects of secondary metabolites may be suppressed during sample preparation by changing the type of isolation buffer or altering its composition (e.g., lowering the pH of the buffer, adding polyvinylpyrrolidone, metabisulfite, β-mercaptoethanol; for more details see Loureiro et al. [14]). However, it is often more convenient to employ an alternative tissue of the plant having reduced contents of

these compounds. Suitable candidate tissues are, for example, petals (e.g., in succulents, Boraginaceae), pollen, roots, seeds, young seedlings, etiolated shoots, and leaf stalks instead of blades (e.g., in Rosaceae species rich in phenolics). In case of flavonoid-mediated interference with FCM staining, reported for pigmented young leaves of *Rumex pulcher* (Polygonaceae; [15]), a selection of alternative tissue (ideally colorless or green) may help to overcome the problem. Kron and Husband [18] introduced the "filter bursting method" for extracting pollen nuclei, which may enable routine and efficient DNA content estimation from pollen in problematic species. Sliwinska et al. [19] demonstrated that FCM analysis of seeds was more successful than analysis of fresh leaf tissue in several species containing interfering compounds, though seeds are not generally recommended for precise genome size estimation (see Dry seeds section).

Some environmental conditions have been found to trigger increased production of interfering secondary metabolites. For instance, increased synthesis of polyphenolic compounds (flavonoids) can be stimulated by increased UV-irradiance, in order to protect DNA from radiation damage [20, 21]. Higher contents of such compounds may even substantially bias genome size estimates using FCM [1]. This was unintentionally shown by Price and Johnston [22] using sunflower plants that were cultivated under different light regimes in a growth chamber. In a follow-up study, the authors clearly demonstrated environmentally triggered synthesis of interfering compounds [3]. Furthermore, pathogen and herbivore attacks often induce increased synthesis of secondary metabolites (chemical defense) by the plant [23], some of which are known to interfere with FCM analysis (e.g., tannins, [17]). The possible effect of environmental variation should be considered during both field sampling and cultivation of plant material for FCM analysis.

1.3 | Pathogens, parasites, and epiphytes

Before sampling material for FCM analysis, a careful visual inspection of the sampled plant is recommended in order to detect possible signs of pathogen and herbivore infestation and/or presence of epiphytic organisms. If overlooked, contaminant nuclei of these organisms co-processed with the measured sample may produce additional peaks in the FCM histograms (depending on the genome size divergence of the sample and its contaminating species). Common sources of such contamination include aphids, thrips, leaf-mining insects, rust fungi, and epiphytic mosses and algae. When undesired organisms are detected, it is convenient to either sample a different leaf on the same individual or carefully clean the leaf surface before processing the sample. Aside from possibly leading to erroneous peak assignments in FCM histograms, pathogen and herbivore attacks often induce increased synthesis of secondary metabolites [23], and may even trigger higher levels of endopolyploidy in plant tissues [9, 12]. Since the plant response may not be restricted to the attacked organs and tissues, excluding strongly infested individuals from field sampling is highly recommended.

2 | TISSUE QUANTITY

The overall amount of plant material needed for a FCM analysis should be estimated empirically. It depends largely on cell size, tissue type (particularly cell wall thickness, intercellular space), quality of material (including tissue preservation method), and on the established number of nuclei to be analyzed per sample. For the recommended fresh leaf tissue, 20 to 100 mg of leaf material (~0.5–1 cm^2) is usually enough to obtain 5000–10,000 nuclei. In general, the yield of nuclei per tissue volume depends on cell size, which usually correlates with genome size [24]. A higher quantity of material thus may be needed when studying plant groups with larger genomes (e.g., ferns, monocots), polyploid taxa, endopolyploid tissues (to ensure sufficient representation of all/target nuclei fractions), or when using plant tissues composed of cells with large vacuoles (e.g., fleshy water-storage tissue). A likely effect of tissue preservation should also be considered as, for example, desiccation of plant material typically leads to a substantial increase in the amount of tissue necessary to release sufficient nuclei for analysis. While the required quantity of tissue should be determined by a pilot FCM analysis before the study is initiated, it may still be subject to between-sample variation due to lower quality of some samples (e.g., wilting leaves, old seeds), tissues exhibiting varying degrees of endopolyploidy, or (hidden) intraspecific ploidy-level variation in a model species. For this and other reasons, it is advisable to use a slightly higher amount of plant material than the minimum. On the other hand, there are also cases, when using a smaller amount of plant tissue can significantly improve the performance of FCM analysis. This is especially true for analyzing tissues with high content of secondary metabolites interfering with FCM measurement. The adverse effect of secondary metabolites can be, to a certain degree, 'diluted' by using a smaller amount of tissue and employing lower chopping intensity during the sample preparation. Finally, an appropriate amount of the internal reference standard tissue should be used to provide FCM histograms with similar nuclei counts for both sample and standard peaks. As with the sample tissue, this amount should be determined empirically.

Given that, in general, small amounts of somatic tissue are sufficient for DNA content estimation using FCM, sample collection does not generally lead to substantial and irreparable plant damage. Thus, repeated analyses can be conducted on the same individual, or via screening individuals at the stage of freshly developed seedlings (e.g., for ploidy level variation and aneuploidy; [25]) using only a part of a cotyledon or a leaf. The small amount of tissue needed and the usually nondestructive sampling make FCM especially suitable for studying rare material (e.g., natural mutants), endangered species, and plants in long-term experiments.

3 | SAMPLING MATERIAL FOR FCM ANALYSIS

In general, fresh plant tissue is preferred for nuclear DNA content estimation and this should be reflected when planning the sampling of

material for FCM analysis. Emphasis should be put on minimizing the time between tissue sampling and conducting FCM measurements. The easiest way to achieve this goal is provided by plant cultivation under controlled conditions at the same facility, but that is often undesirable (e.g., when documenting processes in natural populations) or difficult. Field collection of fresh plant material, on the other hand, imposes many additional challenges (see below), such as great emphasis on short-term storage of samples during their transport to the laboratory, more pronounced impact of environmental variation, and, in some cases, the need to obtain collection permits and phytosanitary certification.

3.1 | Ex situ cultivation under controlled conditions

Cultivation of plant material for FCM analysis under controlled conditions is in many aspects the most suitable approach, provided it is feasible with regards to cultivation requirements and growth rates of the studied plants, expected sample size, and availability of cultivation facilities. Aside from effectively minimizing the time lag between sampling and FCM analysis, cultivated plants (or tissue cultures) provide a stable source of additional material in the case that some samples need to be re-analyzed. Growing the plant material to be sampled under constant and controlled conditions minimizes potential environmental biases and may help attain greater quality of FCM measurements. Additionally, pathogen and herbivore infestations may be more easily prevented and contained under controlled conditions. As outlined in previous sections, environmental factors may affect the quality of sample tissue, for example, via triggering increased content of secondary metabolites or increased levels of endopolyploidy. The most important parameter in this respect seems to be light intensity (particularly UV-B levels), however, effects of water availability and soil quality were also reported (i.e., drought- and salinity-induced stress; [3, 9, 12, 13]). For the high reproducibility of published FCM estimates, it is always important to carefully and comprehensively report the conditions under which the plant material was raised.

Ex situ cultivation is particularly suitable if the main focus of a study is a between-group comparison (e.g., comparing genome size/endoreduplication patterns across systematic groups or species). A major drawback limiting the use of ex situ cultivation as a universal source of plant material for FCM analysis is its problematic application for documenting rates of ecological and evolutionary processes occurring in natural populations. Considering that individuals under study are typically grown from field-collected seeds, their analysis may not reflect the same patterns (e.g., cytotype composition, proportion of hybrid individuals) as those observed at the level of adult individuals from natural populations [25]. As a matter of fact, the diversity observed at the level of seeds may often be a substantial overestimate, since it will include unfit phenotypes that would be quickly lost from natural populations by selection.

3.2 | Field collection and transport to the lab

Field collection of material raises several issues of particular importance: (a) more profound variation in tissue quality due to environmental variation, (b) potentially greater risk of plant misidentification (making voucher specimens is highly recommended!), and (c) suitable storage conditions for fresh-tissue samples during their transport to the laboratory, or implementation of an appropriate material preservation strategy (see next section).

When sampling representative plant materials in the field, any individuals showing signs of strong pathogen or herbivore attack, exhibiting very atypical morphology (compared with rest of the population), or growing in extreme conditions should be omitted from material collection, unless such phenotypes are the primary aim of the study. In that case, however, stringent analysis criteria should be kept to avoid potential biases caused by, for example, increased secondary metabolite content which is likely in such individuals. In general, all recommendations related to the putative effect of environmental factors on the quality of FCM estimates (discussed in the previous section) are also valid for the field-collected material; however, the researcher is usually unable to control this environmental variation and can only attempt to reduce its effect on sampled individuals. Regarding the overall amount of plant material to be sampled, it is usually convenient to collect much more than needed (unless, e.g., sampling endangered species). This approach both compensates for quality decay of material during transport to laboratory and provides the opportunity of re-analyzing some samples if the quality of FCM histograms turns out to be insufficient.

Several alternatives exist for the storage and transport of field-collected plant material: (a) maintaining freshly-sample tissues under cool and humid conditions for up to 2 weeks, (b) transporting generative or vegetative propagules (seeds, fruits, bulbs, tubers, and rhizomes), (c) employing long-term preservation strategies—desiccation, freezing or chemical fixation of tissues, and (d) yet rarely used, isolating nuclei in field followed by their storage in protective solutions such as glycerol. As the best strategy is often species-dependent, it is highly recommended to compare the performance of different approaches and their various adjustments to reach it. However, whenever possible, use of fresh plant material should be preferred, as this approach usually enables high-quality FCM measurements. After harvesting, the plant material is usually wrapped in paper towels slightly moistened with water, and enclosed in a plastic resealable bag [1]. Other variations include wrapping the material in moist filter paper and enclosing it either in a normal plastic bag or in aluminum foil. While the main purpose is to maintain sufficient humidity to keep the sampled tissue fresh, overly wet conditions may encourage tissue degradation and rotting. To reach an optimal balance, it is often convenient to keep some air inside the bag. Sometimes, putting greater amounts of plant material into the bags may be sufficient to maintain the humidity level without the need for moistened paper. Plant material that is prone to wilting, such are thin or finely-dissected leaves, may be more efficiently transported as a short part of leafy stem (or as whole plants with roots), with only the bottom part of stem (or roots) wrapped in moist paper. Another important factor is the sample temperature during transport.

Ideally, the samples should be cooled, for example, using portable refrigerating units; if coolers with ice or cooler packs are used, then padding should be placed between samples and ice to avoid cold damage to the tissue. The samples should be, even over the course of sampling, kept in shade and particularly out of direct sunlight. When the sampled materials are enclosed in plastic bags, even a short exposure to direct sunlight may irreversibly damage (burn) the sampled tissue.

It is not always possible to transport fresh-tissue samples to the laboratory within 1 or 2 weeks, especially when sampling in remote regions without easily accessible FCM facilities, and the time to FCM analysis may be further extended if the laboratory is working at saturation or is experiencing periods of instrument maintenance. The delivery time across continents for both priority postal and express parcel services is frequently well beyond the optimum for transport of fresh plant tissue. Delays at international borders are often imposed at customs, and the material under study may also require phytosanitary certification. Higher success of material transport may be achieved by using specialized plant reproductive propagules, such are seeds, fruits, bulbs, tubers or rhizomes. Out of these, seeds are predominantly being used for their convenient (often long-term) storage, and FCM analysis can be conducted already on dry seed material ([19]; see below), germinating seedlings [25], or older grown plants [26].

Another possibility is provided by long-term preservation strategies, such as desiccation, freezing, or chemical fixation of tissues (see below, Table 2). For the most commonly applied tissue desiccation, a frequently used procedure is as follows: in the field, at the point of

TABLE 2 Advantages, limitations, and potential applications of different material storage and preservation strategies in plant FCM

Plant material	Advantages	Limitations	Potential applications
Fresh tissue	• Easy sampling • Reliable FCM measurements (fluorescence stability, high resolution)	• Need for immediate FCM analysis (short cold storage is possible) • Challenging transport (cold space, phytosanitary certificate may be required)	• Large-scale screening of ploidy variation across populations and species • Genome size estimation in absolute units • Detection of small differences in the amount of nuclear DNA
Silica gel-desiccated tissue	• Convenient sample preservation and transport • Long-term storage (months, sometimes years) • Possibly lower interference of secondary metabolites (e.g., in some Rosaceae)	• Possible decrease in fluorescence intensity • Lower resolution than in fresh tissue	• Large-scale screening of ploidy variation across populations and species (preferably DAPI staining) • first insights into genome size variation
Dry seeds	• Easy sample transport • Convenient and long-term storage (months to years) • Detection of apomixis (FCSS; 69)	• Need for a proper timing of collection • Possible shift in fluorescence intensity • Higher rates of hybrid, aneuploid or odd-ploidy individuals than among adults • Nonrepeatable analysis (the entire seed is usually spent)	• Screening of ploidy variation across populations and species (preferably DAPI staining) • Inferring sexual/apomictic origin of seeds (mating system studies)
Freshly germinated seedlings	• Reliable FCM measurements (fluorescence stability, high resolution) • Easy sample transport (seeds) • Convenient and long-term seed storage (months to years)	• Need for a proper timing of collection • Possible problems with germination • Higher rates of hybrid, aneuploid, or odd-ploidy individuals than among adults	• Screening of ploidy variation across populations and species • Genome size estimation in absolute units • Detection of small differences in the amount of nuclear DNA
Frozen tissue	• Convenient sample preservation • Long-term storage (years)	• Basic lab facility needed (freezer) • Challenging sample transport • Likely shifts in fluorescence intensity • Lower resolution than in fresh tissue	• Screening of ploidy variation (preferably DAPI staining) • First insights into genome size variation
Chemically fixed tissue	• Long-term storage (months)	• Challenging selection of an (also preserved) internal reference standard • Likely shifts in fluorescence intensity • Lower resolution than in fresh tissue	• Screening of ploidy variation (preferably DAPI staining)
Glycerol-preserved nuclei	• Reliable FCM measurements (fluorescence stability, high resolution) • Long-term storage (months)	• Basic lab facility needed • Inconvenient sample transport • Challenging selection of an internal reference standard	• Screening of ploidy variation • Genome size estimation in absolute units • Detection of small differences in the amount of nuclear DNA

sampling, fresh tissues are enclosed in separately-labeled tea bags (or any other material that is well-permeable to air). Several samples in their bags are then pooled and placed either into a plastic resealable bag or an airproof plastic box filled with silica gel in sufficient amounts to lead to rapid desiccation of the sampled material. See Kron et al. [27] for sampling and desiccation of pollen that has several specifics. This process of desiccation typically occupies a few days to 1 week for completion. In contrast to transporting fresh tissues, pooling excessive amounts of plant materials can substantially slow the desiccation process and thus decrease the resulting quality of samples. Occasional shaking with the bag/box is highly recommended during the first hours and days of desiccation to redistribute the silica-gel beads, ensuring the silica-gel beads surrounding plant material are not saturated with humidity. Using silica gel with humidity indicators is very convenient for visual inspection, since the silica gel may need replacing during the desiccation process depending on the initial water content of the samples. After drying, the samples can be stored for longer periods in plastic boxes containing small amounts of silica gel, either at room temperature or frozen. Tissue preservation using chemical fixatives (ethanol- or formaldehyde-based), although widely used in animal and human FCM studies, have attracted only little interest from the community of plant biologists [28].

4 | MATERIAL PRESERVATION STRATEGIES

As a general rule, tissue preservation is unlikely to improve the quality of results as compared with those obtained with fresh tissue, and this should be considered in the initial study design. Importantly, particular preservation strategies differ substantially in their advantages and limitations (Table 2). It is accepted that good quality results can be obtained using dried tissue, but if a species or tissue is problematic when fresh material is used, it will almost certainly perform even more poorly when dried (a point first made by Suda and Trávníček [29]). On the other hand, the use of preserved tissues does open entirely new avenues for research in remote and understudied geographical areas, such as in the tropics [30]. Nonetheless, genome size estimation using dehydrated plant material should be avoided if at all possible. If that it is not the case, then the effects of drying on fluorescence intensity (hence DNA content) should be rigorously assessed either on a subset of samples or in a separate calibration data set (e.g., [31]). Unless the authors can present supporting results, no claims implying that their FCM estimates from preserved tissue are fully comparable to those obtained from fresh material should be made.

4.1 | Desiccation

There have been several attempts to replace fresh plant samples with dry (or frozen) somatic tissue over the last two decades. For example, Suda and Trávníček [29, 32] described the use of DAPI staining for reliable ploidy level estimation in desiccated plant material (either silica- or air-dried), and this method was successfully applied to a number of plant groups (e.g., [33–38]). The protocol can be also extended to recently collected herbarium vouchers [39, 40]. Most of the analyzed plants yielded distinct peaks after several months of storage at room temperature, with fluorescence intensity of nuclei isolated from desiccated tissues and stained with DAPI being highly comparable to that of fresh material. As desiccation is a routine way of field sample preservation, the possibility of using dehydrated tissues opens new and promising prospects for plant FCM. On the other hand, there are also some disadvantages to the desiccation approach. There may be a decrease (up to 10%) in fluorescence intensity compared to that of fresh samples (e.g., [32, 39, 41, 42]) frequently resulting in lower reliability of such DNA content estimates. Decrease in the uniformity of fluorescence was also observed [32], leading to higher CVs (coefficients of variation) of the peaks and more prominent background noise. The quality of FCM measurements also decreases with the aging of desiccated samples, even though no significant decline in quality was observed after 9 months, and for majority of tested species also after 20 months [29]. The quality decay in desiccated samples can be slowed down by their deep-freezing (−80°C), prolonging the possible storage time up to several years [29].

These observations are not compatible with the high standards required for some applications, including the determination of absolute genome size [1, 15, 43]. Consequently, the majority of published studies have used preserved material solely to determine DNA ploidy levels, which can tolerate some relaxation of the quality criteria (e.g., [35,44–50]). In case a minor variation in DNA content is expected, it is advisable to calibrate the dried samples by a subset of samples analyzed from fresh tissues (e.g., [31]) to account for potential drop in the quality of analysis. In a carefully designed experimental study, Bainard et al. [42] evaluated the potential use of silica gel-dried plant material with PI staining in plant genome size research. The authors concluded that sample desiccation introduced comparatively minor variation (<10%), a level of which was species-specific and comparable to other sources of artefactual variation. While this study has shown the prospects of using PI FCM with desiccated sample tissue, its routine use for absolute genome size estimation should still be avoided.

Additionally, plant organ and tissue selection seem to act as important predictors of successful DNA content estimation from desiccated material. For example, species with soft and thin leaves were those unsuccessfully analyzed among the sets of 21 silica gel-dried tropical species [51] and 60 air-dried temperate species [29]. A preliminary screening across several alternative tissue types is highly recommended for assessing the effect of tissue desiccation on quality of FCM analysis and selecting the optimal tissue. For example, analysis of desiccated petals delivers results of higher quality as compared with desiccated leaves in many Brassicaceae species. Desiccated pollen may be a good choice when it is difficult to get high-quality results with somatic tissue and also for its convenient storage [18, 27]. However, a thorough evaluation of the performance of different dry plant tissues (organs) for DNA content estimation using FCM is still lacking, thus general recommendations cannot be provided at this time.

4.2 | Freezing

Reasonable FCM histograms have been achieved by analysing rapidly-frozen plant tissues [39, 52–54], either for estimation of absolute [55–57] or relative nuclear DNA contents [53, 58]. Frozen plant material has also been successfully used for establishing DNA synthesis patterns in developing seeds of Norway maple [59]. When considered as an alternative to the transport of fresh material from the field, frozen material may be more challenging for both sample preparation and transport. Rapid freezing (e.g., using liquid nitrogen) of the sampled tissue and maintaining it in the frozen state until FCM sample preparation is strongly recommended. Moreover, FCM analysis of frozen tissue frequently results in lower-resolution histograms when compared to those prepared from fresh samples and a quality decay of samples with their aging is also apparent. In spite of the possible shifts in fluorescence intensity, the suitability of this storage strategy for genome size estimation remains largely unstudied. Until any targeted studies are made, the use of frozen tissues should be considered with caution, preferential applications including initial ploidy-level screening in model species or obtaining the first insights into genome size variation.

4.3 | Chemical fixation

Chemically fixed somatic tissue (either by nonadditive fixatives such as ethanol-acetic acid, or additive ones, such as formaldehyde) has long been considered inappropriate for absolute DNA content measurements using FCM, being only useful, ultimately, for screening of ploidy variation in model species. So far, few authors have used non-additive fixatives [60–62], the main reason being that the protocol involves enzymatic digestion of cell walls, and thus it is a laborious and time-consuming approach. In addition, the use of pectinases and cellulases for cell-wall digestion may later result in decreased fluorescence intensity of nuclei [63]. Finally, it seems highly probable that DNA staining by intercalating dyes (e.g., PI) can be disturbed by chemical fixation. The possible reasons are either a direct modification of chromatin structure by the fixative or the release of tannins from vacuoles, which strongly bind to chromatin and interfere with quantitative DNA staining, as was shown for the Feulgen reaction [64]. This strongly argues against the use of chemically-fixed tissue in research projects involving absolute nuclear DNA estimation [65]. Even though Overton and McCoy [65] were able to completely reverse the effect of formalin by re-suspending formalin-fixed human cells in PBS and heating them at 75°C for at least 1 h prior to staining with PI, such approach was unsuccessful with plant material. Specifically, neither re-suspending in PBS nor heating restored completely the fluorescence of *Pisum* and *Glycine* leaf tissue nuclei that were previously fixed in 4% formaldehyde and stored for 24 h [66]. Further limitation of the use of fixatives is the challenging selection of a suitable reference standard for a sample of unknown genome size, as the reference standard should be subjected to the same preservation procedure.

4.4 | Storage of isolated nuclei in protective solutions

A promising alternative to physical and chemical preservation of plant tissues when high-quality analysis is required represents the storage of isolated nuclear suspensions in protective solutions such as glycerol. The use of glycerol for preservation of isolated nuclei for FCM analysis was first mentioned by Chiatante et al. [67], who stored purified nuclei of pea in 30% glycerol at −20°C for several months. A more thorough assessment of the method was conducted by Hopping [68], who stored isolated nuclei of *Actinidia deliciosa* in 30% glycerol at −20°C and discovered FCM estimates highly comparable with those obtained from fresh samples (only a 5%–7% decrease in fluorescence) after 9 months of storage. However, the first rigorous statistical evaluation of this approach across multiple plant species was provided in time-scale laboratory and in situ field experiments by Kolář et al. [51], also allowing direct comparisons with the performance of fresh and silica-gel dried material. This study demonstrated that plant nuclei preserved in ice-cold Otto I buffer together with a 60% glycerol solution remained intact for at least a few weeks when kept at −18°C, and provided estimates of nuclear DNA content that were highly comparable with those obtained from fresh samples analyzed immediately after collection. Recently, Kobrlová et al. [30] successfully employed this material preservation strategy to study genome size variation in tropical flora of Borneo, reaching high precision FCM measurements of glycerol-preserved nuclei (mean CVs of both sample and standard nuclei <3%) after a month of storage.

The protocol is compatible with both DAPI and PI staining and not only allows ploidy level determination but also genome size estimation in absolute units, including the detection of small differences in the amount of nuclear DNA. Despite the higher laboriousness, glycerol-preserved nuclei apparently represent the most reliable way of sample preservation for genome size research. Limitations of the method include the necessary in situ sample processing, the challenging transport of samples (frozen at −18°C), and difficulties with standardization (i.e., selecting a suitable standard without the previous knowledge of sample genome size, as well as having some in situ). The problems with standardization can be overcome by preparing the same sample repeatedly with different internal standards differing considerably in their genome size [30], though this substantially inflates the number of samples to be analyzed.

4.5 | Dry seeds

Over the last two decades, several investigators have used dry seed material for determination of nuclear DNA content by FCM with a considerable success (e.g., [19, 69–73]). A mature seed of sexually reproducing angiosperm plants typically has several components: (a) the 2C embryo, (b) the 3C endosperm, specialized storage tissue that may also contain endopolyploid cells, and (c) the 2C seed coat, which usually consists of dead cells and thus remains undetected in a FCM histogram [74, 75]. Different patterns can be observed in

apomictic seeds, where the endosperm tissue may be 2C, 4C, 5C, or 6C, depending on the type of apomixis [69]. In gymnosperms, the overall seed structure is very similar but with a 1C endosperm. Consequently, when whole seeds are analyzed, not only the targeted G_0/G_1 embryo nuclei but also endosperm nuclei are detected, leading to the presence of additional peaks in FCM histograms. The embryo may take up more than 75% of the seed volume or, in other cases, constitute less than 25% of its volume [75]. Especially in the latter scenario, the low proportion of embryo to endosperm nuclei in the FCM sample can easily lead to erroneous peak assignments. The situation may be even more challenging when either embryo or endosperm nuclei peaks overlap with that of internal reference standard. Extracting embryo out of the seed or removing parts of the seed that do not contain embryo are recommended solutions. An additional complication is the presence of endopolyploid tissues in some embryos. Dissecting a specific part of the embryo with prevailing 2C nuclei (usually the radicle; [19]) may facilitate interpretation of resulting FCM histograms. In some plant species, the DNA content estimates from dry seeds and leaves were highly comparable [19]. Moreover, in the case of woody plant members of Rosaceae with leaves containing high amounts of secondary metabolites interfering with fluorescent staining, the analysis of seeds provided more reliable results [72]. It is possible that dormant embryonic tissues with low water content also release less nucleases and secondary metabolites into nuclear homogenate than typical fresh tissues [15]. On the other hand, due to different degrees of chromatin condensation, the seeds should be generally avoided for precise genome size measurements. Only viable seeds with intact embryo nuclei are suitable for nuclear DNA content estimation. While dry seeds are preferable for sample preparation, short soaking in water may facilitate extraction of embryo from the seed and initiate DNA repair mechanisms (thus possibly improving the precision of DNA content estimates). Nevertheless, soaking seeds for longer periods will decrease the quality of FCM measurements due to high metabolic activity in germinating seeds. Seed sample preparation may otherwise follow the standard razor blade-chopping method; however, sample incubation for 10–60 min is highly recommended (the exact time is species dependent and should be established empirically) as it usually improves the quality of measurement (see, e.g., [76] for a detailed procedure). In addition, while the addition of RNase is optional for most tissues, it is necessary in the case of seeds [14].

There are several advantages to using seeds for nuclear DNA content estimation. Seeds are convenient for easy transport of plant material, and allow long-term storage, and the analysis of dry seeds saves cultivation costs. On the other hand, their use may be discouraged by the need for collecting plant material only during the seed gathering season, potential differences in genome size estimates from seeds as compared to fresh leaves (e.g., [19]), inability to repeat the FCM measurement (entire seeds are usually spent for each analysis) and in some cases also by the necessary knowledge of seed biology (e.g., for embryo dissection). Another drawback, most manifested in taxonomically challenging plant groups, is that field collected (open pollinated) seeds may contain a higher proportion of hybrid, odd-ploidy, or aneuploid individuals than is the case among adult plants in the same populations, that is, variants that otherwise would soon be removed by natural selection (e.g., [25]). Thus, in order to get a representative DNA content estimate for a population, multiple seeds per plant/population should be analyzed.

5 | SAMPLE STORAGE

Plant material can be stored for up to months prior the FCM analysis of nuclear DNA content. The shortest storage time is typical for fresh material, ranging from several days to 2 weeks, which mainly depends on the tissue type and storage conditions (temperature and humidity levels). On the other hand, specialized plant reproductive propagules, such are bulbs and tubers may allow a longer storage (months) and in the case of seeds, the storage time may be prolonged to years, especially when these are stored frozen under anoxic conditions. Frozen samples of fresh plant material can be stored up to months or years for ploidy level determinations; dehydrated tissue can be preserved for months or even years (herbarium specimens: [33, 77]; silica gel-dried samples up to 2 years old: [45]). Finally, isolated nuclei in glycerol suspensions can be stored for up to weeks or months. However, ex situ cultivation of plant material (including also in vitro cultures; e.g., [78]) is still the best long-term source of fresh tissue for most rigorous FCM applications.

6 | BEST PRACTICES

1. Fresh and not fully expanded leaves are a recommended first-choice plant material for FCM analysis. Any sampled tissue should be intact, and parasite- and pathogen-free.
2. The overall amount of plant material required for a FCM analysis should be estimated empirically, as it depends on tissue type, its quality (including material preservation method) and plant genome size. During field sampling, it is often convenient to collect more material than needed, thus compensating for quality decay during transport to laboratory, and providing an opportunity to re-analyze some samples.
3. In most cases, long-term material preservation techniques decrease the quality of FCM measurements (compared with fresh tissue). Advantages and limitations of particular preservation techniques should be considered in context of the research aims. For a ploidy screening, desiccated samples are usually the best compromise between quality of analysis and convenience of transport and storage. On the other hand, seedlings (obtained from collected and transported seeds) or nuclei isolated in the field and stored in protective solutions are well suitable for accurate genome size estimates.
4. Whenever possible, reduce the time lag between tissue sampling and conducting FCM analyses. This is also relevant when long-term material preservation strategies are employed.
5. In case of low-quality measurements (or their difficult interpretation) due to a presence of cytosolic compounds interfering with fluorescent staining and/or endopolyploidy, we highly recommend

alternative tissue selection (different organs, possibly at different stages of development) as the first step in protocol optimization, followed by changing a buffer or its composition.

6. Be aware of the putative effect of environmental factors on tissue quality (e.g., reflected in the content of interfering cytosolic compounds), which can be manifested both in natural conditions and under experimental cultivation. Special emphasis should be given to light intensity (mainly UV-B irradiance levels).
7. Tissue type and quantity, sampling, and transport details (incl. Material preservation), as well as environmental conditions under which the plant material for FCM analysis was cultivated (e.g., light and irrigation regime, soil quality) should be carefully reported in manuscripts.

ACKNOWLEDGMENTS

This study was supported by the Czech Science Foundation grant 20-22783S (Filip Kolář), by the long-term research development project RVO 67985939 of the Czech Academy of Sciences (Martin Čertner, Magdalena Lučanová, Filip Kolář), by Charles University Research Centre program No. 204069 (Martin Čertner), by the CENTRO2020 projects CULTIVAR (CENTRO-01-0145-FEDER-000020) and RENATURE (CENTRO-01-0145-FEDER-000007), and by Fundação para a Ciência e a Tecnologia (UIDB/04004/2020) (João Loureiro).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Čertner M, Lučanová M, Sliwinska E, Kolář F, Loureiro J. Plant material selection, collection, preservation, and storage for nuclear DNA content estimation. Cytometry. 2021;1–12. https://doi.org/10.1002/cyto.a.24482