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Received: 12 November 2020 | Revised: 1 February 2021 | Accepted: 24 February 2021

DOI: 10.1002/cyto.a.24331

REVIEW ARTICLE

ISAC CYTOMETRY Journal of Quantitative Cell Science PART A

Isolation of plant nuclei for estimation of nuclear DNA content: Overview and best practices

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Funding information
Centro2020, Grant/Award Numbers: CULTIVAR project (CENTRO-01-0145-FEDER-000020), Project RENATURE (CENTRO-01-0145-FEDER-000007); Fundação para a Ciência e a Tecnologia, Grant/Award Number: UIDB/04004/2020; Natural Sciences and Engineering Research Council of Canada, Grant/Award Number: RGPIN-2020-05652

Abstract

A critical aspect for obtaining accurate, reliable, and high-resolution estimates of nuclear DNA content is the release of nuclei from the cytoplasm in sufficient amounts, while maintaining their integrity throughout the analysis, protecting their DNA from degradation by endonucleases, and enabling stoichiometric DNA staining. In embryophytes, the most common method consists of chopping the plant material with a sharp razor blade to release nuclei into an isolation buffer, filtering the homogenate, and staining the nuclei in buffered suspension with a fluorochrome of choice. Despite the recent description of alternative approaches to isolate nuclei, the chopping procedure remains the most widely adopted method, due to its simplicity, rapidity, and effectiveness. In this review article, we discuss the specifics of nuclei isolation buffers and the distorting effects that secondary metabolites may have in nuclear suspensions and how to test them. We also present alternatives to the chopping procedure, options for filtering and fluorochromes, and discuss the applications of these varied approaches. A summary of the best practices regarding the isolation of plant nuclei for the estimation of nuclear DNA content is also provided.

KEYWORDS
best practices, embryophytes, flow cytometry, genome size, nuclear isolation, nuclear suspensions, plant sciences, ploidy level

Plants differ from animals in a few general ways, one of which is the presence of a cellulosic cell wall. In addition, since embryophytes (land plants) comprise complex three-dimensional tissue architectures of interlinked cells, it seemed that flow cytometry (FCM), whose requirement for single-particle suspensions is mandatory, was not applicable to embryophytes [1]. However, the first report that used this technique already dates back to 1973 [2]. The author used a cytometer called "Impulscytophotometer" to analyze fluorescence signals from field bean nuclei prepared from fixed tissues after enzymatic treatments with pectinase and pepsin. The methodology used was laborious and time-consuming, and the fact that the article was written in German may have hampered a higher impact in plant research [3].

It was only ~10 years later that FCM started to be applied more frequently in plant sciences. After discarding the possibility of using intact cells for estimating DNA content (the rigid cell wall is autofluorescent and confers an irregular shape to cells that disturbs the hydrodynamic flow within the cytometer), researchers focused on protoplasts (the cell wall being removed using hydrolytic enzymes) that are spherical and behave regularly within the flow stream [4]. However, the low permeability of the plasma membrane, the autofluorescence of cytoplasm and chlorophyll, and the "off-center" position of the nucleus hindered the production of histograms of fluorescence with adequate resolution [5]. If the first two obstacles could be circumvented by fixation of protoplasts with ethanol-acetic acid, the irregular position of the nucleus remained a problem. Therefore, efforts were implemented to isolate intact nuclei for flow cytometric analyses. In 1983, two different methods to isolate intact nuclei were proposed: Puite and Ten Broeke [4] obtained intact nuclei after

protoplast lysis in the presence of non-ionic detergents, while Galbraith et al. [6] prepared a suspension of nuclei by chopping a small amount of fresh tissue in a hypotonic buffer supplemented with a non-ionic detergent. Despite leading to very good histograms for estimating DNA content, the first approach was laborious, time-consuming, and inapplicable to species and tissue types for which protoplasts could not be prepared [3]. Therefore, few researchers subsequently have employed this method (but see References 7–11). In contrast, the ingenious method developed in the laboratory of David Galbraith was simple, convenient, rapid, and capable of providing histograms of high quality for many plant species. This method largely stimulated the application of flow cytometry in plant sciences, and it remains the main and most reliable procedure for nuclear isolation from tissues of embryophytes. Parenthetically, beyond plants, the chopping approach to produce nuclear suspensions has found wide applicability in the cytometric analysis of nuclei of species in many other eukaryotic kingdoms, including examples from insects, mammals, fish, and fungi.

1 | NUCLEI ISOLATION BUFFERS

The composition of the nuclear isolation buffer is one of the most important aspects for obtaining accurate, reliable, and high-resolution estimations of nuclear DNA content. Considering the diversity in tissue structure and chemical composition in plants, it is not surprising that no single buffer is optimal for all species [12]. However, certain isolation buffers consistently provide better results than others, at least when model species are analyzed. So far, more than 30 different buffers for nuclei isolation (or simultaneous isolation and staining) have been described. Greilhuber et al. [13] summarized the chemical composition of 10 of the most popular noncommercial buffers, which collectively accounted for about three-quarters of published FCM papers at the time [14]. In 2006, Loureiro et al. [15] introduced two new buffers, the general-purpose buffer (GPB) and a woody plant buffer (WPB), that have been well received, with an average of 28 citations per year. From the more than 30 isolation buffers available in the literature, Galbraith's buffer [6], $MgSO_4$ buffer [16], LB01 [17], Otto's [18, 19], Tris·$MgCl_2$ [20], and WPB [15] account for the majority of FCM publications. There are also several commercial buffers sold by cytometer manufacturers (e.g., Partec, now Sysmex) that often give good results, and thus have been used in some laboratories [14]; however, their formulation is not always publicly available.

An isolation buffer is made of several common components: (a) **organic buffer substances**, for example, MOPS [6, 21], Tris [17, 20, 22], or HEPES [16, 23]—these components control the pH of the solution, usually set at near-neutral pH (from 7.0 to 8.0) compatible with common DNA fluorochromes, and keeps the nuclei in an intact state; (b) **non-ionic detergents**, for example, Tween-20, Triton X-100—these components release and clean the nuclei, and decrease the tendency of nuclei and debris to aggregate; please note that ionic detergents, such as sodium dodecyl sulfate (SDS) change the fluorescence properties of the dye molecule, and thus are not used [24]; (c) **chromatin stabilizers**, for example, $MgCl_2$, $MgSO_4$, spermine; (d) **chelating agents**, for example, EDTA, sodium citrate—these components bind divalent cations, which serve as nuclease cofactors, thereby blocking DNase activity; and (e) **inorganic salts**, for example, KCl, NaCl—these components provide an appropriate ionic strength [3]. Furthermore, some buffers are enriched with mercaptoethanol, metabisulfite, ascorbic acid, or dithiothreitol as reductants, and polyvinylpyrrolidones (e.g., PVP-10 or PVP-40) to bind phenolics (see below).

Compared with the more complex buffer compositions described above, Otto's buffer [18, 19, 25] combines two solutions in a two-step procedure: the first comprises citric acid plus a detergent, usually 0.5% Tween-20, for nuclei isolation (Otto I), with the second, basic $Na_2HPO_4{\cdot}12H_2O$ plus fluorochrome (Otto II), being added to the isolated nuclei to enable staining at neutral pH and to increase its salt concentration. The Otto II solution is often supplemented with 2-mercaptoethanol and, in some laboratories, the fluorochrome is not added until after Otto II. The final pH value of the mixture is determined by the volume ratio of the two components (1:2, Otto I: Otto II, is often used; however, in most cases, this corresponds to a 1:4 ratio since about half of the Otto I volume is lost during the chopping and filtration steps); this system is also known as McIlvaine's buffer [25]. Therefore, Otto's buffers differ from other buffers as the first step combines nuclei isolation with slight fixation and some histone removal and the second step stabilizes the nuclear solution. When Otto's buffers work, they usually provide histograms of very high quality, evidenced as low coefficients of variation (CV) of the fluorescence peaks. For example, Otto's buffers plus DAPI were used for the first time in unfixed plant nuclei by Doležel and Göhde [19] for sex identification in *Melandrium*, attaining CVs of G_0/G_1 peaks of 0.5%–0.7%. As the isolated nuclei are usually stable in the Otto I solution (although this behavior can be genus- or family-specific), it is often possible to prepare several samples in advance. However, caution is needed: there are some species for which nuclear samples deteriorate quite quickly in Otto I buffer, and therefore must be stained after only 1–2 min. Recent modifications of Otto's buffers, published by Šmarda et al. [26], include increasing the acidity of Otto I buffer (by addition of hydrochloric, nitric, or acetic acid), and/or the use of different detergents at different concentrations. These changes improved the release of nuclei from particularly problematic tissues, such as those containing mucilage.

The different buffer characteristics and the secondary metabolites released upon chopping the plant material can affect the quality of the sample and of the estimation itself. For example, Loureiro et al. [12] compared four widely-used and chemically different isolation buffers, namely Galbraith's buffer [6], LB01 [17], Otto's buffer [18, 19, 25], and Tris·$MgCl_2$ [20], using a set of plant species that covered a wide range of genome sizes (1.30–26.90 pg/2C) and that differed in tissue structure and composition. Each buffer performed differently, although acceptable results were obtained in most cases. Excellent results (a combination of high fluorescence yields, high nuclei yields, little debris, and low CV values) were obtained only for some species with some buffers. Therefore, the best choice of the buffer is species-dependent, and thus, when starting to analyze a new species, it is

recommended to test more than one buffer. From this work, it was also evident that some ingredients generally seem to work better than others. Spermine seemed to be a better chromatin stabilizer than MOPS/$MgSO_4$. Tris does not buffer well at or below pH 7. A higher concentration of non-ionic detergent (0.5% Triton X-100) was fundamental for the improved performance of Tris·$MgCl_2$ buffer in species with high levels of mucilage. The results obtained in this comparative work were the basis of the proposal of two new buffers, GPB and WPB [15]. The chemical composition of GPB was based on that of LB01, with Tris being replaced by MOPS. Moreover, the concentration of Triton X-100 was raised to 0.5%, which reduces the attachment of debris to the isolated nuclei. As woody plant tissues are typically rich in phenols and other secondary metabolites, a new buffer, WPB, that could deal with these compounds was developed. WPB is based on the composition of the Tris·$MgCl_2$ buffer but includes a chelating agent and inorganic salts, and Triton X-100 at 1.0%. Sodium metabisulfite and PVP-10 were also added to handle the presence of secondary metabolites. In species relatively free of secondary metabolites, GPB provided similar and, in some cases, better results than WPB, and may be preferred. When compared with other nuclear isolation buffers, the use of WPB seems to result in improved histogram quality and, thus, its use was recommended as the first choice when problematic tissues/species are to be analyzed.

2 | EFFECTS OF SECONDARY METABOLITES

A key aspect that should be considered when selecting an isolation buffer is the way it copes with the distorting effect of secondary metabolites. The interference of these compounds with staining, irrespective of the fluorochrome, has long been recognized in cytophotometry [27] and, since 2000, specifically in plant FCM [28, 29]. This interference was considered to lead to fluorescence inhibition, but recent research has revealed additional effects, including aggregation of minor particles with nuclei that also play a part in this interference and can even lead, in some cases, to an apparent increase in nuclear fluorescence [13, 30]. Because of this effect, Greilhuber et al. [13] distinguished between staining inhibitors and coatings of debris. First, staining inhibitors provide steric barriers to fluorochrome binding and thereby result in the reduction of fluorescence. This presumably results in a left-hand shoulder or tail, or a shift of the whole peak to the left, if all nuclei are equally affected. Such a tail may be confluent with non-nuclear aggregates. Second, secondary metabolites may bind to nuclei and then attract fluorescing debris, creating a halo of low-fluorescing particles around nuclei. This coating of debris creates a right-hand tail on, or shoulder to, the nuclear peaks (easily visualized inside light scatter [SSC] vs. PI scatterplots, Figure 1A) and affects sample and standard nuclei in the same way without necessarily leading to a decrease in the overall nuclear fluorescence (Figure 1B, C) [13, 30]. It is also possible that very large polymeric polyphenols do not penetrate the nuclei but only attach externally, thus leading to a slight increase rather than a decrease in fluorescence intensity [13].

Phenolic substances possessing active hydroxyl groups are among the most problematic fluorescence inhibitors. Condensable tannins and the hydrolyzable tannins (e.g., tannic acid, ellagic acid) are some of the widely known types of phenols for which distorting effects have been reported; coumarins have also been associated with variable DNA value measurements in *Bituminaria bituminosa* [31]. When nuclear suspensions turn brown or display precipitation, the presence of phenolics is evident, and the results of the analysis of these samples should be treated with caution. In these situations, the addition to the isolation buffer of antioxidants, such as mercaptoethanol, ascorbic acid, or sodium metabisulfite is highly

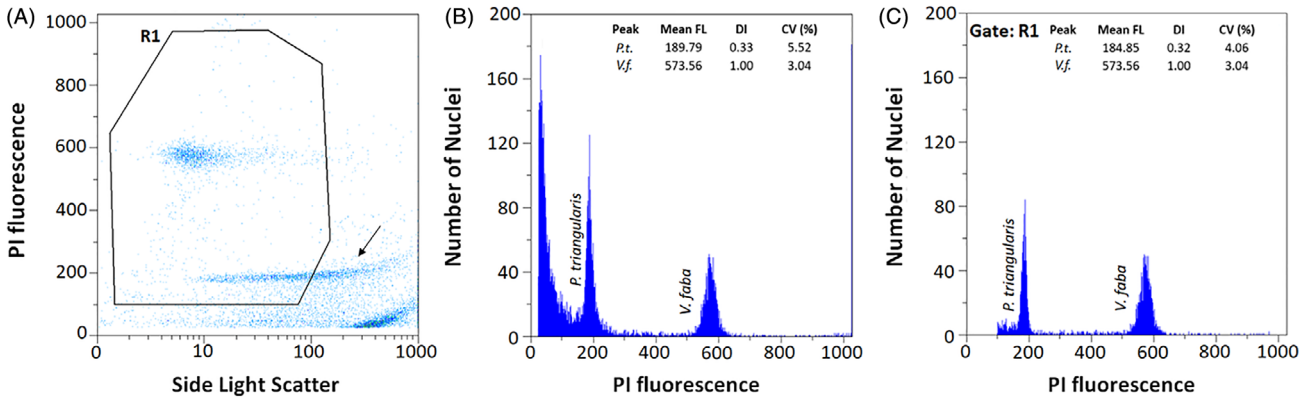

FIGURE 1 Use of gating to eliminate the distorting effect of secondary metabolites in propidium iodide (PI) stained nuclei isolated from leaves of *Pentagramma triangularis* (*P.t.*) and *Vicia faba* (*V.f.*, as reference standard). Gating (region R1) was used in the side light scatter (SSC) versus fluorescence scatterplot (A) to exclude particles at the right-hand tail or shoulder (indicated with an arrow) that represent coating of debris as a result of secondary metabolites binding to nuclei and leading to the attraction of fluorescing debris. PI fluorescence histograms before (exhibiting unsatisfactory quality, B), and after gating (C) are also presented. Please note that despite the right skewing of the DNA peaks before gating, the mean fluorescence of each G_0/G_1 peak was not affected, and thus the DNA index (DI = mean channel number of sample/mean channel number of reference standard) is very similar before and after gating. WPB was used as buffer and nuclei were stained with 50 $\mu g \cdot ml^{-1}$ of PI for 5 min. The mean channel number (mean FL), DNA index (DI), and coefficient of variation (CV, %) value of each G_0/G_1 peak is also given

advisable in order to keep phenolics in their reduced states. Any hydrogen bonds could then hopefully be maintained in their reversible state and be disrupted by the addition of a competitor. PVPs, for this purpose, are the most used competitor; for reasons of viscosity, the lower molecular weight classes (e.g., PVP-10, PVP-40) are used in FCM. PVPs can reactivate enzymes which have been inactivated by phenolics [32]. The combination of PVP with antioxidants in nuclear isolation buffers, to allow the phenolics to be stripped from proteins and DNA before they become oxidized, has been shown to improve histogram quality in high-phenolic species [33, 34], and therefore is highly recommended. The effect of the cytosol on propidium iodide (PI) fluorescence was also demonstrated by Noirot et al. [28, 35–37] in *Coffea*. Cytosol from *Coffea* leaves and defined components such as chlorogenic acid, led to a reduction in the fluorescence yield of *Petunia hybrida* nuclei, which was employed as the non-phenolic control. Addition of caffeine was able to partly restore the fluorescence yield of quenched petunia nuclei [36], which may be explained by the known gallotannin-binding property of caffeine.

In 2006, Loureiro et al. [30] applied tannic acid in 13 concentrations to nuclear suspensions of *Pisum sativum* prepared with four buffers. With increasing tannic acid concentration, nuclei to which debris of low fluorescence was attached could be visualized. This caused an uneven increase in fluorescence intensity and SSC among nuclei. The SSC disclosed a fraction of nuclei with attached debris as a tail, the so-called "tannic acid effect", which could be visualized in a SSC versus PI fluorescence scatterplot, as populations of nuclei with flag shaping (e.g., Figure 1 showing this effect in nuclei of *Pentagramma triangularis*). At the highest tannin concentrations, general precipitation of the sample was observed.

But, how best to test for the distorting effect of secondary metabolites? First, implement the routine analysis (Figure 2) of SSC versus PI and/or forward light scatter (FSC) versus PI scatterplots, and look for signs of a "shoulder" (as in Figure 1A), and plot fluorescence versus time to monitor whether the fluorescence intensity is stable over the time course of measurement (Figure 2). This will help to detect the "tannic acid effect." Second, as previously mentioned, pay particular attention to the color of the nuclear suspension. If it turns brown, or if some precipitation is evident (PI itself might also be precipitated; this is visible when the precipitate turns pink or reddish), it is highly probable that the results (the fluorescence histogram and related scatterplots) will be affected. Third, a simple test, as proposed by Price et al. [29], can be followed, that is, compare the fluorescence intensity of the standard nuclei isolated alone with that of standard nuclei isolated together with the unknown sample using the same method (i.e., the same quantity of plant material, buffer, chopping intensity). If the fluorescence intensity of the co-chopped standard appears to have changed (either, reduced or increased) compared with the standard chopped separately, this difference is likely an effect of the secondary metabolites released from the sample tissue. This test is particularly important when the quality of the histograms is good but there is a fluorescence intensity shift, which is the case when the concentration of the secondary metabolites is not high enough to lead to the coating of debris. In other rarer cases, despite the peaks seeming of poor quality and skewed, the fluorescence peak positions of both sample and standard may be stable. In such cases, either modal values instead of the means can be taken, or rigorous gating applied. When the clean nuclei population can be sorted out based on the SSC versus PI fluorescence scatterplot, analytic tools can be used (e.g., gating) and physical purification of nuclei is not necessary. However, the users should describe in detail how this gating was applied, including examples of the gated regions as illustrated in Figure 2, accompanied by the test results involving the inhibitors.

What, then, are the best practices recommended for buffer selection and avoidance of the distorting effect of secondary metabolites? To start, it is possible that a specific buffer will not give consistently good results for every species [3]. Although WPB and Otto's buffers are recommended as the first choices, it might be worth testing various buffers to identify the best one for a given plant material. Another

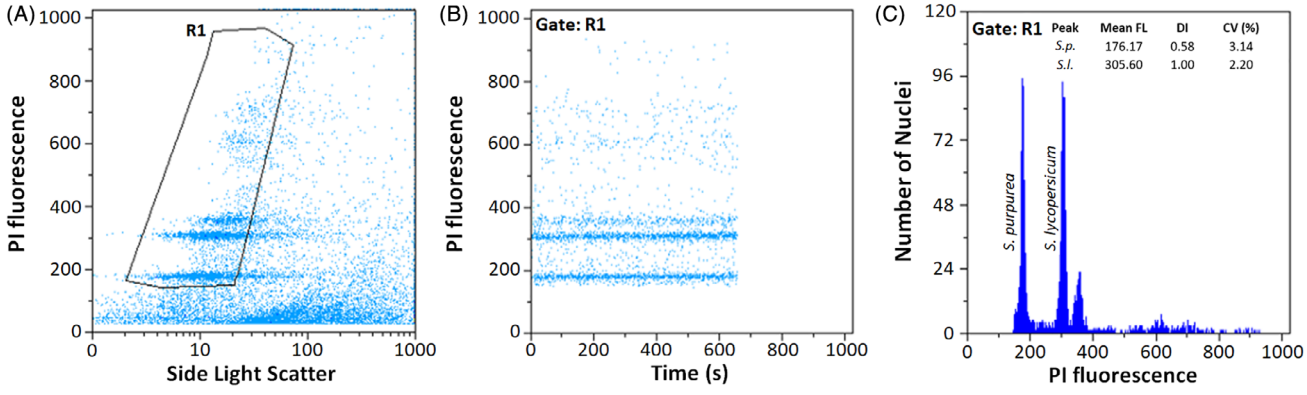

FIGURE 2 Display set-up with routine gating (region R1) for improved resolution of propidium iodide-stained nuclei, comprising side light scatter versus propidium iodide (PI) fluorescence (A), PI relative fluorescence versus time (B), showing that fluorescence is stable and does not change with time, and PI fluorescence histogram (C). Nuclei were isolated from *Spergularia purpurea* (*S.p.*) and *Solanum lycopersicum* (*S.l.*, as reference standard), WPB was used as buffer and nuclei were stained with 50 $\mu g \cdot ml^{-1}$ of PI for 5 min. The mean channel number (mean FL), DNA index (DI = mean channel number of sample/mean channel number of reference standard) and coefficient of variation (CV, %) value of each G_0/G_1 peak is also given

related best practice is to routinely add antioxidants and PVP to isolation buffers, rather than just resorting to them when problems are detected. Among the most common antioxidants, metabisulfite has been shown to perform well while being much less toxic than mercaptoethanol; therefore, its use is also recommended. Optimal fixation (in Otto I when using Otto's buffers) and staining times should be tested for the given material, as well as chopping intensity. When samples turn brown or produce precipitates, this can be taken as an indication of "over-chopping" or of chopping too much plant material. Also, tissues with lower levels of interfering secondary metabolites can be tested (e.g., very young leaves, cotyledons, or leaf petioles, although some of these tissues tend to be endopolyploid, so care must be taken to correctly identify peaks). Other suggestions include cooling all devices and buffer solutions (e.g., by working on ice, or in a walk-in cold room), as low temperatures will slow down undesirable chemical reactions. Some proportion of contaminants may be removed by inserting a centrifugation step into the protocol: isolate the nuclei, filter (to remove large particles), centrifuge at low speed (e.g., 150 g for 5 min, [38]), discard the supernatant and re-suspend the nuclei in fresh buffer. If problems with secondary compounds persist after various optimization attempts, reduce the amount of material to the minimum required to obtain a sufficient number of nuclei needed for the analysis (this number may vary depending on the application, for example, a total of 5000 nuclei for genome size estimation when the peaks are of similar height and the background is low [38]), since such practice also reduces the concentration of contaminants released into the homogenates. Last, as the effects of secondary metabolites cannot be completely eliminated, the good practice is to guarantee an equal effect of any secondary metabolites on the sample and standard nuclei. This is achieved by chopping both materials in a sandwich-like fashion rather than sequentially, because during sequential chopping, the nucleus and inhibitor in the same cell are co-localized, while the standard nuclei are influenced by diluted inhibitors. This was verified by Bennett and co-authors [39] when analyzing poinsettia (*Euphorbia pulcherrima*) comprising green leaves and red bracts rich in phenolics, with the level of PI staining, obtained being strongly influenced by the arrangement of tissues when chopped sequentially.

3 | CHOPPING AND ALTERNATIVE METHODS OF SAMPLING PREPARATION

As indicated above, the first method to isolate intact nuclei was to lyse protoplasts in a hypotonic buffer. In this manner, the nuclei are gently released from cells, and the procedure usually provides histograms of DNA content with a little background and DNA peaks having very low CVs. However, protocols for protoplast isolation are laborious, and are available only for a limited number of species and for particular tissues at certain developmental stages. Therefore, a universally accepted method for the preparation of nuclear suspensions for DNA FCM was developed by Galbraith et al. [6]. In this protocol, cell nuclei are released from a small amount of fresh plant tissue by chopping it with a razor blade in an appropriate buffer solution. After filtration of the homogenate through a nylon mesh or commercial filter and the addition of a DNA fluorochrome (if it was not previously added to the nuclei isolation buffer), the sample is ready for analysis. The method is rapid (3–5 min for experienced users), convenient and suitable for many (though not all) plant species, organs, and tissues. Variations of Galbraith's et al. protocol have been introduced, which differ in the way a sample is homogenized and by the composition of nuclear isolation buffers. Although originally developed for fresh plant tissues, Galbraith et al. protocol is also suitable for the estimation of relative nuclear DNA content in dry herbarium vouchers [40], silica-dried samples [41], and seeds [42]. In the case of seeds, the same buffers as for leaf tissue can often be used, but in some species, other buffers perform better, such as the seed buffer [43] developed specifically for this purpose.

In the chopping procedure, it is important that the razor blade or scalpel is sharp, and the material is chopped into very fine pieces and not just crushed. Although some laboratories use each edge of the razor blade only once, depending on the type of plant material (dried or xeromorphic material will dull the razor blades faster than soft tissue) and on the material of the Petri dish (glass tends to reduce sharpness more rapidly than plastic), each blade can be used for as long as it is able to cut the plant material efficiently into fine pieces.

In plant species with mucilaginous compounds, as these compounds tend to attract to sample and standard nuclei and to never release them to the filtered isolate, Dobeš et al. [44] developed a method that includes the use of an ultrasonic water bath step after chopping that assists freeing the nuclei.

Tilting the Petri dish so that less buffer is concentrated in a smaller region of the dish allows the plant material to be sufficiently moistened, but not completely submerged in the buffer (which would impede efficient chopping). This can be achieved through a slight slope created with a rolled piece of paper or chopping can be done in only a half volume of the buffer, with the other half added after chopping and before filtration. Some authors have suggested that doing the chopping in a walk-in cold room (with all components chilled to 4°C) can be helpful in plant samples with high levels of secondary metabolites [5]; however, this is not a common practice in most plant FCM laboratories and chopping samples on ice may be a simpler solution.

Given a lack of standardized rules regarding chopping intensity and duration, the extent of chopping should be determined empirically. As discussed above, chopping at too high intensity may lead to problems related to debris and secondary metabolites. Therefore, it is advisable to adjust the chopping intensity to the plant material and species under study. At first, rather than trying to obtain high nuclei numbers, it is advisable to use small tissue amounts and minimal chopping, and if good quality peaks with low nuclei numbers are obtained, the amounts of tissue and of chopping can be increased until the required number of nuclei (depending on the application) is reached.

Alternative options to release nuclei from various tissues include the "bead beating" method. Bead beating is the release of nuclei from the tissue by mechanical agitation in a liquid containing glass, metal, or ceramic beads 2–3 mm in diameter. This high-throughput method of sample preparation has been successfully used with a variety of

tissues, including dry or fresh leaves [45–49], pollen [50, 51], and lycophyte and fern spores [52]. General approaches include the use of tissue homogenizers that hold relatively few samples [47, 49], 96-well plate homogenizers [53], and for pollen and spores, standard vortexers [51, 52]. When direct comparisons have been made with chopping, results have indicated that sample CV values are usually similar for the two methods, while nuclei numbers may increase with bead-beating [47, 48, 53]. This result, in combination with increased speed, ease of preparation, and, potentially, reduced cost, make bead-beating an appealing option. Nevertheless, the similarity in quality between bead-beaten and chopped samples can be species- and tissue-dependent, and some authors have reported increases in debris levels ranging from inconsequential [48] to problematic [52, 54]. The use of the method may therefore be limited by quality concerns, and quality control criteria need to be provided in the methods and results sections of any publications.

The preparation of nuclei from pollen grains usually requires a specialized isolation method [55]. Due to their particulate nature and rigid wall, most pollen grains escape unopened when chopped with a razor blade. Loureiro and Castro [56] presented an overview of many pollen grain nuclei isolation methods, with the method of Kron and Husband [54] of bursting pollen through a nylon mesh being identified as the most promising approach. Release of nuclei from seed tissues has also been described by crushing between two sheets of fine sandpaper [57].

4 | REMOVAL OF DEBRIS FROM A SAMPLE

The almost universal method for filtration of the nuclear homogenate after chopping employs nylon mesh or various commercial filters with pore sizes ranging from 20 to 50 μm (species with larger genome size requiring larger pore sizes). Filtration removes cell/tissue fragments and large debris. In some applications, such as extraction of nuclei from pollen, the use of filters with a smaller pore size (10–20 μm) may be preferable [54].

Lee and Lin [58] suggested an alternative approach for recalcitrant tissues containing calcium oxalate crystals. These authors designed a cotton column that after wetting in the isolation buffer was rolled and inserted in the middle of a 5 ml pipette tip. This method apparently reduced the percentage of contaminants and, in this manner, purified the nuclear suspension. The only disadvantage was a 50% decrease in nuclei yield.

In some cases, centrifuging nuclei may assist removal of cellular debris and secondary metabolites (see above in section "Effect of secondary metabolites"). For example, in leaf homogenates of high viscosity, a series of centrifugation steps may help to clean the samples. However, this approach also tends to reduce nuclei yield [38].

5 | STAINING

After sample homogenization, nuclei must be stained with a fluorochrome, which should bind specifically and stoichiometrically to DNA. Fluorescent stains can be divided according to the binding mode to DNA as follows: (a) dyes that quantitatively intercalate into double-stranded nucleic acids, and (b) base-specific minor groove-binding dyes with AT- or GC-selectivity. The fluorochromes used so far in plant DNA FCM are listed in Table 1.

For DNA measurements in absolute units (pg of DNA or Mbp), it is essential to use intercalating dyes (propidium iodide, PI or ethidium bromide, EB), as these fluorochromes are not affected by DNA base composition [59]. For maximal fluorescence and highest resolution, EB and PI should be added at near-neutral pH (between 7.2 and 7.4) and at saturating concentrations [60–62]. EB also requires a buffer that provides ionic strength (i.e., with KCl or NaCl, [63]). In addition, pH values below 7 appear to enhance the activity of endonucleases [60, 61]; however, studies on the effect of pH on staining intensity, histogram quality, and DNA specificity in plant FCM are urgently needed. Although Barre et al. [62] suggested that the optimal dye concentration should be determined for each given pair of species, concentrations between 50 and 150 $\mu g \cdot ml^{-1}$ are usually found appropriate [13, 30].

Unfortunately, EB and PI are sensitive to the chromatin structure [64, 65], which implies that changes in chromatin condensation (due to growth state, tissue type, or secondary metabolites) might affect DNA content estimations. As discussed above, this susceptibility is the main reason why tissue fixation is not recommended for absolute DNA content estimations. For reasons that remain unclear, PI has been considered superior to EB [66], possibly due to its lower toxicity, making PI the preferred intercalating dye for staining plant nuclear DNA.

As PI and EB bind to double-stranded RNA (dsRNA), pretreatment with ribonuclease (RNase) has been considered necessary for meaningful DNA measurements. However, RNase addition may often show no effect due to a low dsRNA content, in leaves for instance, and thus may appear dispensable; still, in tissues rich in RNA, such as meristems and seeds, this might not be the case. Indeed, preliminary analyses using a standardized protocol with a large nuclear homogenate obtained from leaves, and further subdivided into several samples with and without the addition of RNase revealed no significant effect of the addition of RNase (data not shown). Therefore, for most applications and tissues, after some preliminary tests, FCM users may opt to avoid this step, reducing the cost and time of sample preparation. However, this should be explicitly mentioned in the methods section of any publication.

Despite the requirement for excitation in the UV range with mercury arc lamps, lasers, and more recently, with LEDs, fluorochromes with AT-specificity (DAPI and Hoechst dyes) are highly popular for relative DNA measurements (e.g., ploidy level estimations), with DAPI clearly being the preferred dye. This is probably because a comparative study between both dyes revealed that DAPI provided histograms with higher fluorescence intensity and resolution [63]. In contrast to what is seen with intercalating dyes, the binding of DAPI to DNA is not influenced by the state of chromatin condensation, which can result in histograms with higher resolution. DAPI (at nonsaturating concentrations ranging from 1 to 10 $\mu g \cdot ml^{-1}$) or Hoechst staining of

TABLE 1 Fluorochromes used in plant DNA flow cytometry

Fluorochrome	Primary binding mode	Wavelength (nm)[a]	
		Excitation	Emission
Propidium iodide (PI)	Intercalating	538 (blue-green)	617 (orange)
Ethidium bromide (EB)	Intercalating	523 (blue-green)	603 (orange)
DAPI	AT-selective	359 (UV)	461 (blue)
Hoechst 33258	AT-selective	352 (UV)	451 (blue)
Hoechst 33342	AT-selective	360 (UV)	460 (blue)
Chromomycin A3	GC-selective	445 (violet-blue)	570 (green)
Mithramycin	GC-selective	445 (violet-blue)	575 (green)
Olivomycin	GC-selective	440 (violet-blue)	560 (green)

Note: Information on the binding mode and excitation and emission maxima wavelengths are also provided.

[a]Excitation and emission spectra of DNA-fluorochrome complex were obtained from ThermoFisher Scientific: https://www.thermofisher.com/pt/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html#!/.

DNA (usually ranging from 2 to 5 $\mu g \cdot ml^{-1}$) is usually carried out at pH 7.0.

Mithramycin, together with other fluorescent antibiotics (chromomycin A3, olivomycin), are fluorochromes that bind to the GC-rich regions of DNA. These dyes are optimally excited at about 440 nm (Table 1), and due to the lower resolution of histograms as compared with AT-specific dyes [10], their use in plant DNA FCM is almost completely restricted to base composition studies. Mithramycin, the most used GC-specific stain, is typically used at concentrations ranging from 50 to 100 $\mu g \cdot ml^{-1}$ [10] and its optimal staining is achieved in the pH range of 5–9 [67]. Also, the presence of magnesium ions is fundamental for the formation of complexes between GC dyes and DNA, with the concentration of $MgCl_2$ (in the range of 15–150 mM) being important for higher fluorescence intensity and histogram resolution [67]. As all base-specific dyes bind only to double-stranded DNA, the addition of RNase is not necessary.

For any of these staining protocols, the fluorochromes can either be already present in the nuclei isolation buffer or be added after nuclear extraction and filtration. While some time can be saved in the first approach, it also increases the probability of human skin and laboratory contamination and creates a higher number of disposables that must be treated as toxic waste [13]. Therefore, the second strategy is highly preferable in most situations.

Stained samples can be maintained at room temperature in buffers containing metal chelators (e.g., EDTA, sodium citrate); room temperature is also suitable for the activity of RNase when RNA digestion is needed. On the other hand, samples prepared in buffers without these components and containing divalent cations (Mg^{2+}, Ca^{2+}) should be incubated at ice-cold temperature to decrease the activity of DNases [67].

An incubation period of 5–20 min is usually adequate for saturating the DNA sites with occasional shaking (when feasible); longer staining times (e.g., 60 min) are not recommended as they often lead to a decrease of fluorescence and/or to increased levels of background debris [62]. However, the optimal staining period for a given plant species and tissue should always be determined empirically. A practical way to make such an evaluation, is to monitor the stability of fluorescence over time (Figure 2B). If the incubation period was not enough for a given species/plant material, it is possible to witness the increase of fluorescence over time, until stability is reached. If this happens, the events recorded until the moment of fluorescence stability should be cleared or the acquisition reset, and the upcoming samples should be incubated for a longer period of time. Gating to record only fluorescence intensities that are stable over time is another alternative. Another factor that may influence the incubation period (to reach the saturation of binding sites by the stain) is the amount of debris present in the sample and the effect of secondary metabolites. Again, any effect of time on nuclei fluorescence, in these cases through a reduction of fluorescence, can be easily monitored and gated in scatterplots showing fluorescence over time.

6 | CONCLUSIONS/BEST PRACTICES

1. Test a range of different buffers when working with a new species and tissue to identify the one that performs best. For newcomers to the plant FCM field, WPB and Otto's buffers are suggested as first choices.
2. Adjust chopping intensity of the plant material being analyzed. Remember that higher intensity is not always the best (and can be compensated by increasing the amount of plant material).
3. For DNA measurements, sample and reference standard must be co-chopped, rather than prepared separately and then mixed.
4. For DNA measurements in absolute units use propidium iodide at saturating concentrations. For DNA estimations in relative units and when higher resolution is needed (e.g., aneuploidy, sex determination), DAPI can be used at nonsaturating concentrations.
5. Consider adding the stain after nuclear homogenization and filtration instead of including it directly in the nuclear isolation buffer.

For Otto's two-step procedure, do not stain immediately after isolation but only after the addition of Otto II.

6. 5 to 10 min is usually enough for the incubation with the fluorochrome. Still, the optimal staining period for a given plant species and tissue should be determined experimentally by examining the fluorescence stability in scatterplots of fluorescence versus time.
7. Be alert to the possible interference of secondary metabolites. Browning and/or precipitation of the nuclear suspensions are common symptoms of the undesirable presence of phenolic compounds.
8. The occurrence of the described "tannic acid effect" should be checked by measurement of the light scattering properties of particles (Figure 1). The test for inhibitors suggested by Price et al. [39] can be another approach for detecting the disadvantageous interference of secondary metabolites.
9. When interference by secondary metabolites is observed, and assuming several nuclear isolation buffers have already been tested, different tissues having lower or no secondary metabolites (e.g., cotyledons, leaf petioles, young stems) should be tested in order to attain unbiased estimations. If the effect still persists, the quantity of plant material and the chopping intensity should be reduced.
10. In publications, carefully report all specific details of the protocol to allow reproducibility of the experiments, including the buffer used and all modifications (concentrations, additives), fluorochrome, staining times, type and amount of tissue used, and nuclei extraction method. Representative histograms and scatterplots, and gating procedures should also be provided (for example as supplementary materials), as indicated in Figure 2.

ACKNOWLEDGMENTS

The authors are grateful to three anonymous reviewers for the helpful suggestions and comments, and to David Galbraith for further editing the English language of the manuscript.

AUTHOR CONTRIBUTIONS

João Loureiro: Conceptualization; funding acquisition; writing-original draft; writing-review & editing. **Paul Kron:** Writing-review & editing. **Eva Temsch:** Writing-review & editing. **Petr Koutecký:** Writing-review & editing. **Sara Lopes:** Writing-review & editing. **Mariana Castro:** Writing-review & editing. **Sílvia Castro:** Funding acquisition; writing-original draft; writing-review & editing.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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How to cite this article: Loureiro J, Kron P, Temsch EM, et al. Isolation of plant nuclei for estimation of nuclear DNA content: Overview and best practices. *Cytometry*. 2021;1–10. https://doi.org/10.1002/cyto.a.24331