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Flow cytometric analysis of pollen and spores: An overview of applications and methodology

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Abstract

Pollen grains are the male gametophytes in a seed-plant life cycle. Their small, particulate nature and crucial role in plant reproduction have made them an attractive object of study using flow cytometry (FCM), with a wide range of applications existing in the literature. While methodological considerations for many of these overlap with those for other tissue types (e.g., general considerations for the measurement of nuclear DNA content), the relative complexity of pollen compared to single cells presents some unique challenges. We consider these here in the context of both the identification and isolation of pollen and its subunits, and the types of research applications. While the discussion here mostly concerns pollen, the general principles described here can be extended to apply to spores in ferns, lycophytes, and bryophytes. In addition to recommendations provided in more general studies, some recurring and notable issues related specifically to pollen and spores are highlighted.

KEYWORDS

best practices, DNA content, flow cytometry, genome size, ploidy level, pollen grains

Pollen grains are the male gametophytes in a seed-plant life cycle. Mature pollen grains are complex in that they have a durable outer layer (sporopollenin impregnated pollen wall) enclosing at least two cells (vegetative and generative in binucleate angiosperm pollen), often three (one vegetative and two sperm cells, in trinucleate angiosperm pollen) [1] and up to five in gymnosperms [2]. Generative and sperm cells are enclosed within the vegetative cell and at certain developmental stages form weak physical associations with the vegetative nucleus, known as male germ units (MGUs) [3]. Vegetative nuclei are often morphologically different from sperm and generative nuclei, with different shapes, sizes, and degrees of chromatin condensation (density) [1,4]. Microspores, the precursors to mature pollen grains, are morphologically similar to sperm or generative cells in the sense that they are relatively simple uninucleate cells.

All of these structures (mature pollen grains, microspores, sperm and generative cells, MGUs, and nuclei) have been the subject of flow cytometric investigation. Less commonly, other pollen inclusions such as vesicles have been studied [5]. While protocols vary by application, they typically include collecting pollen or spores and in many cases extracting their contents (Section 1.1), analytically circumscribing events of interest using cytometrically obtained data (e.g., gating; Section 1.2), and, in some cases, physically separating particles of interest with a sorter (Section 2.1).

1 | IDENTIFICATION AND ISOLATION OF STRUCTURES OF INTEREST

1.1 | Pollen collection, extraction, and release of its contents

Whether testing whole pollen or its component parts, the first consideration is the collection of sufficient quantities with as few contaminants

as possible. Depending on the species and the sample size requirements of the study, collecting sufficient quantities of pollen will range from straightforward (e.g., most wind-pollinated species) to difficult and time-consuming, as in small-flowered selfing species producing low amounts of pollen (e.g., *Arabidopsis thaliana*), for which collection of large numbers of flowers and even dissection may be required. Collection of microspores has the further complication that they must first be released from flower buds by some form of tissue disruption, followed by multistep purification, for example, see References 6–8.

In many species, direct collection of loose pollen is not practical, and instead, collecting structures ranging from anthers to whole inflorescences may be necessary, often followed by drying (to release pollen from anthers, as well as for storage) and some process of filtering out non-pollen tissue. As an example, flowers or their parts can be put into open Eppendorf tubes in tube holders inside an airtight box or bag with silica gel, and allowed to dry over 1–2 days, with pollen released into the tubes. The filtering process typically involves the suspension of collected structures in an isotonic liquid, vortexing, and filtering through an appropriate-sized mesh. Such procedures may be complex, involving multiple steps and collection buffers designed to minimize pollen bursting [9]. Choice of filter size is critical: too small a filter may exclude larger pollen grains, which may be the very subject of interest (e.g., male unreduced = 2*n* gametes), while too large a filter may fail to remove enough contaminating tissue. For most plant species, the optimal mesh size ranges from 50 to 100 μm, although it may range from 20 to 200 μm. Preliminary measurement of pollen diameter is crucial, usually with a microscope [4].

Contaminating somatic tissue may not be problematic in some applications, but it can be in others, such as the estimation of 2*n* gamete frequencies [10, 11] or tissue-specific gene expression studies [12]. In such cases, careful flower dissection may be necessary, or flow cytometry (FCM) sorting may be used to further purify samples (Section 2.1). Post-measurement, analytical correction for somatic tissue can also be applied in some cases (Section 2.4).

While in some applications, the whole pollen or spore is the structure of interest, in many others the extraction of pollen contents (cells, nuclei, and MGUs) is required. A variety of methods have been used, as reviewed in Reference 4. Chopping is commonly used, probably because it is the predominant method for other tissues, and can be quite effective, particularly with germinated pollen, in which the pollen tubes help to hold the pollen grains in a stable mass and nuclei within pollen tubes are now outside the lysis-resistant pollen wall [3]. Filter bursting, in which pollen grains are gently pressed against a mesh filter, was shown to be more efficient than chopping in a wide range of species [4], and has started to be used more frequently [10, 13–17]. The use of buffers that cause osmotic bursting also works in some species, and can produce very clean, low debris samples [18], although pollen of some species is very resistant to bursting in this way. Bead beating using a tissue homogenizer [19] or simply by vortexing with glass beads [9], has been promoted as fast and efficient but can produce large amounts of debris [4, 20], as does sonication [4, 21]. It is likely that the best method is species-specific, so method testing and comparison is recommended.

Following the release of pollen contents, samples are typically filtered through a small enough mesh to allow passage of pollen contents while excluding the (frequently autofluorescent) pollen wall (typically 10 to 20 μm). Note that even the most efficient application of these methods may only extract contents from a relatively small proportion of all pollen, and some protocols involve repeatedly applying the bursting procedure to intact pollen washed from the filter [9]. Efforts aimed at maximizing nuclei number may result in the majority of sperm cells being ruptured [22], so if sperm cell collection or measurement is the goal (Section 1.2.2), this should be a consideration in choosing an extraction method.

1.2 | Analytic isolation of structures of interest

Analytic isolation refers to the process of identifying and circumscribing (e.g., gating) the particles of interest using FCM data, for example, by gating the events of interest in such a way as to exclude debris and other nontarget particles. Although the parameters involved vary by structure and application, such gating frequently involves the use of side scatter (SSC), forward scatter (FSC), or more than one fluorescence wavelength (Figure 1). Some analytic isolation in this sense is done in most FCM studies, either to generate measurement data for a subset of all particles or to provide triggering criteria for physical sorting (Section 2.1). Methods and criteria for identification and analytic isolation of different pollen structures are described below.

1.2.1 | Whole mature pollen and spores

Within species, whole pollen or spores will typically exhibit relatively low variation in size (measured by pulse width or FSC) and granularity (SSC). Gates created using biparametric scatterplots of these parameters can therefore be used to circumscribe mature pollen, spores, or microspore events before measurement, counting, and/or sorting [9, 23]. Natural autofluorescence of sporopollenin in the pollen wall can also be used for taxonomic identification and sorting of environmental pollen samples (e.g., airborne pollen [24]) and fossil pollen from sediments [23, 25]. While such autofluorescence is relatively uniform within species, users should be aware that exceptions have been observed [26]. Gating based on fluorescence may be further enhanced in the case of fluorescently-tagged lines (FTLs) [27], membrane-permeable DNA stains [9], or the latter combined with SSC variation [28]. Reference samples should be tested to ensure that gating criteria correspond to the observed characteristics of the objects of interest [9].

1.2.2 | Nuclei, generative, and sperm cells

The contents of burst angiosperm pollen grains will include vegetative nuclei as well as sperm or generative cells (depending on whether the

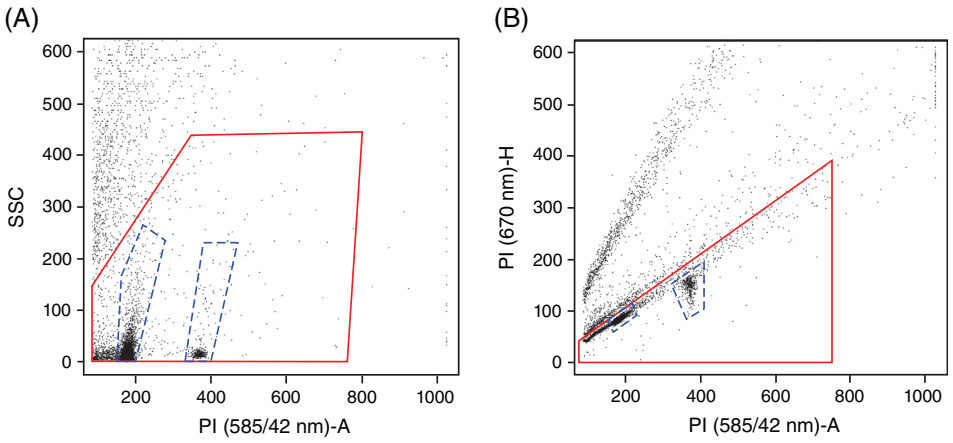

FIGURE 1 Examples of the gating of PI-stained pollen nuclei, using biparametric scatterplots: (A) plot of side scatter (SSC) vs. fluorescence area, PI (585/42 nm)-A, and (B) fluorescence height at a second wavelength, PI (670 nm)-H, versus fluorescence area. Large polygonal regions (solid lines) exclude higher SSC and PI (670 nm)-H debris from the main nuclei regions enclosed. Dashed regions indicate closer gating of 1C and 2C nuclei clusters

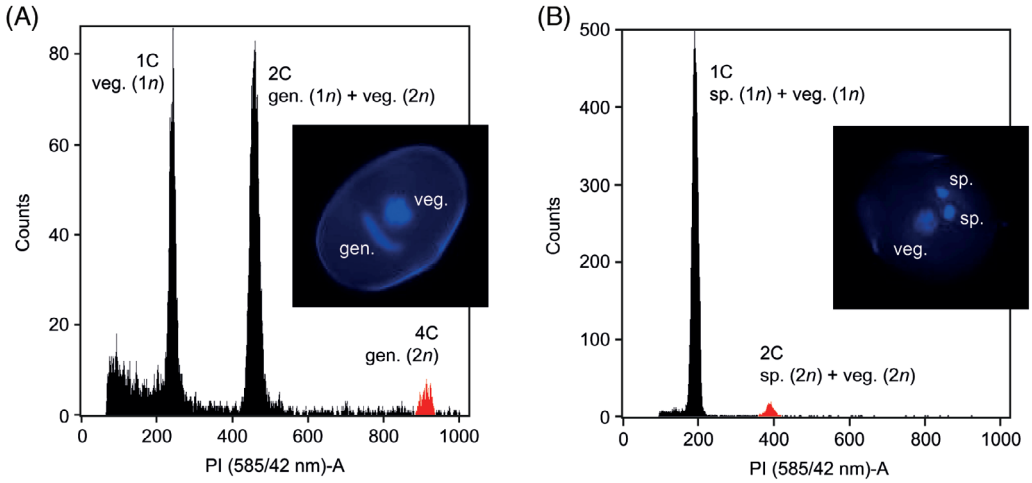

FIGURE 2 Fluorescence histograms of pollen nuclei from (A) binucleate and (B) trinucleate angiosperm pollen. Note that in the context of this figure, the terms "1n" and "2n" refer to products of reduced and unreduced microspores, respectively. (A) 1C events are vegetative nuclei from 1n pollen, 2C events are primarily generative nuclei from 1n pollen but also include 1n vegetative doublets and vegetative nuclei from 2n pollen; 4C events (in red) are generative nuclei from unreduced (2n) pollen and generative doublets from 1n pollen. 3C events, when present, are presumably doublets. (B) 1C events are comprised of vegetative and sperm nuclei from reduced (1n) pollen; 2C events (in red) are vegetative and sperm nuclei from 2n pollen and 1n doublets. Insets show (A) typical binucleate angiosperm pollen, stained with DAPI, with a vegetative nucleus and a generative nucleus, (B) typical trinucleate angiosperm pollen, stained with DAPI, with one diffuse vegetative nucleus and two compact sperm nuclei

pollen is mature and whether it is trinucleate or binucleate at maturity). It will also contain sperm or generative nuclei that have been released from their cells [22]. If the DNA-specific fluorochrome used to stain nuclei is membrane impermeable, such as propidium iodide (PI) or Sytox orange, nuclear fluorescence may only be detected for those sperm or generative nuclei that have escaped their cells. On the other hand, nuclei still contained within cells will fluorescence when membrane-permeable stains are used (e.g., SYBR green, DAPI). Staining with a combination of membrane-permeable and impermeable dyes allows one to distinguish intact sperm cells from free nuclei, permitting the measurement or sorting of the subpopulation of interest [9]. If the objective is to measure nuclear DNA content (fluorescence), regardless of whether nuclei are still enclosed with cells, then nuclei populations can simply be gated based on fluorescence from commonly used dyes (PI, DAPI; Figure 1).

Vegetative nuclei can also be distinguished from sperm and generative cells and their nuclei. In binucleate species, typically containing a 1C vegetative nucleus and a 2C generative nucleus [4], the distinction between the types is obvious based on DNA content (PI or DAPI fluorescence; Figure 2A). In trinucleate species, all nuclei are 1C (Figure 2B), but fluorescence differences between vegetative and sperm nuclei have been observed when certain dyes are used (SYBR green and PI), with sufficient difference to separate the types, at least in some species [9, 14, 22]; Figure 3. These staining differences may be due to the staining of RNA, the quantity of which can differ between vegetative and sperm nuclei [22], and/or to differences in chromatin density between nuclei types [4, 22]. Because vegetative nuclei may be larger, less compact, and differently shaped than sperm and generative nuclei, differences in SSC and FSC may also be used to distinguish them (Figure 3). An alternative approach for trinucleate

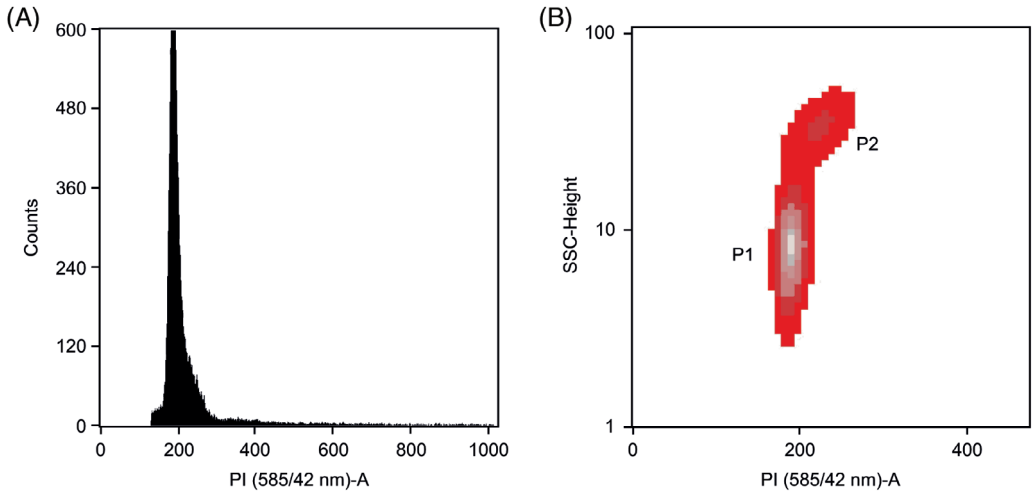

FIGURE 3 (A) Fluorescence histogram of nuclei from trinucleate pollen, showing a shoulder on the right side of the 1C peak; (B) density plot of fluorescence versus SSC for 1C events from (A), showing the presence of two populations of nuclei, possibly corresponding to sperm (P1) and vegetative (P2) nuclei. Note that differences between the two nuclei types are based on both fluorescence and scatter properties

species uses transgenic lines with the nuclei-specific expression of fluorescent proteins (e.g., GFP, RFP), allowing separation of the sperm and vegetative nuclei with high precision, as was successfully demonstrated for *Arabidopsis thaliana* [6]. When vegetative and sperm nuclei are distinguished, counts in FCM samples are often observed to be lower than expected for vegetative nuclei relative to other pollen nuclei types. This may be because they are more easily retained within the pollen wall, accidentally filtered out by a poor filter choice, or more prone to disruption during sample preparation [4, 22]. This should be kept in mind for studies of vegetative nuclei versus other nuclei types [14].

1.2.3 | Male germ units

There are relatively few studies of MGUs using FCM. There has been interested in recent years in MGUs, both for their own sake [3] and for their possible role as false positives in estimates of *2n* gamete nuclei [11, 29]. Careful combinations of microscopy and FCM have demonstrated that MGUs are detectable in FCM samples as 2C events in trinucleate pollen [3]. MGUs represent a kind of aggregate and may be identifiable as such (Sections 1.2.4 and 2.4).

1.2.4 | Aggregates

Aggregates are usually not the target of study: they are a complicating particle type that should ideally be removed. This is a particularly important concern in studies of *2n* gamete production, when pairs of nuclei from 1*n* gametes can be mistaken for nuclei from 2*n* gametes (Section 2.4). Aggregate discrimination using signal pulse analysis is a robust method for identifying and excluding aggregates, and may be used both for pollen contents (e.g., nuclei) and whole pollen grains. Briefly, signal width (time of flight) or signal height (maximum pulse intensity) are plotted against fluorescence area (integrated fluorescence) to distinguish between single particles and doublets that share the same total fluorescence, but differ in maximum brightness (signal height, lower in doublets) or size (signal width, higher in doublets) [11, 30, 31]; Figure 4. Other variants also exist, such as the use of FSC instead of fluorescence area [9]. While this method works well in many cases, it fails for particles that deviate too much from spherical, such as elongated nuclei that may be present throughout whole plant taxonomic groups, and limitations on this method should be well-understood and clearly discussed in publications [11]. For other options for doublet correction see Section 2.4.

2 | RESEARCH APPLICATIONS IN POLLEN FCM

Applications falling under the broad heading of "pollen flow cytometry" vary according to the structure of interest, the parameter being measured, and the objectives of the study. We discuss five broad categories of application, each with its own specific methodological considerations, but note that individual studies may include aspects of more than one category.

2.1 | Sorting and recovery of pollen and its components

This application refers specifically to the use of FCM as a tool to complete a preliminary step in a study: the physical isolation of whole pollen grains, cells, or nuclei for further testing. The particle type (population of interest) is identified analytically using the approaches described in Section 1.2. Sorting is not described in detail here, but

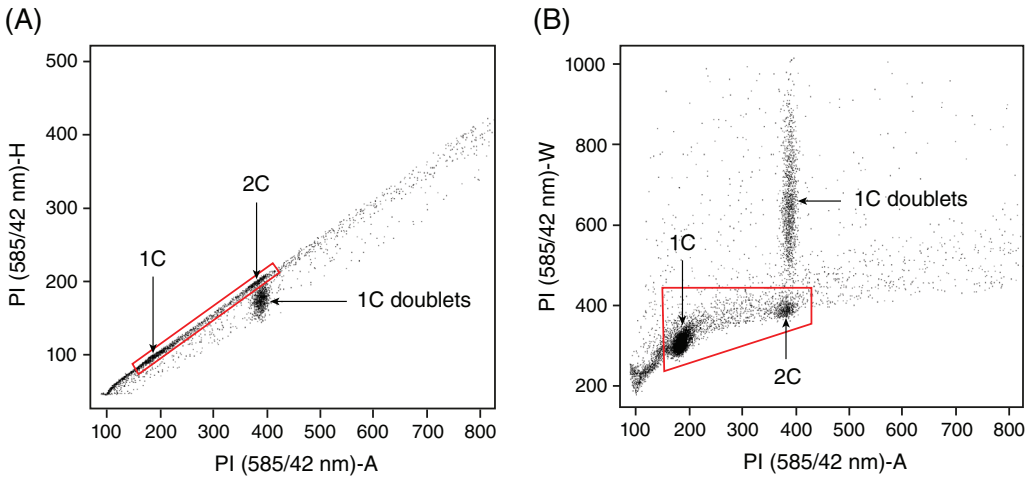

FIGURE 4 Signal pulse analysis for aggregate correction using (A) fluorescence signal height, PI (585/42 nm)-H, or (B) fluorescence signal width, PI (585/42 nm)-W, of PI-stained nuclei from trinucleate pollen. Signal height or width are plotted against fluorescence area, PI (585/42 nm)-A. Arrows indicate clusters of single 1C and 2C nuclei as well as clusters of 1C + 1C nuclei doublets. Polygonal regions (red lines) drawn around regions of single nuclei exclude the doublets that would be mistaken for 2C nuclei

briefly, in an instrument with sorting capability, a gate is created around the population of interest, and particles matching the criteria imposed by the gate are physically recovered for further study. Following sorting, FCM may be further applied to the isolated particles, as described in subsequent sections.

FCM sorting of pollen and its subunits has been used for a variety of purposes. One group of applications is the separation of different types of cells or nuclei followed by a between-type comparison of gene expression or DNA-methylation patterns. With such aims, Pauls et al. [7] used FCM sorting of microspores to discriminate and collect embryogenic cells triggered by high-temperature treatment in *Brassica*, following the protocol previously established in References 8, and Misra et al. 12 used fluorescent activated cell sorting with the transgenic line of *Arabidopsis* to collect individual sperm cells. The FCM sorting of fossil pollen from sediments has found an increasing use in paleoecology. Since the pollen cells are long dead, these applications rely mostly on natural autofluorescence of sporopollenin in the pollen wall or whole pollen light scatter properties (SSC and FSC), which may allow their assignment to a particular (group of) plant species [23, 32]. Promising applications include using flow sorting to obtain fossil pollen samples in sufficient concentration and purity to allow either radiocarbon dating of layers in a sedimentary sequence [23, 25] or extraction and sequencing of ancient DNA [33].

Because the objective of flow sorting is to obtain the correct particle type in as pure a form as possible, methods should be validated using samples of known composition. Recovery of some particle types may be more difficult than others; for example, vegetative nuclei may be more prone to disruption than sperm nuclei [22]. This should be kept in mind if counts of different particle types are an important end product. Instrument settings, including flow speed, must be optimized for the particle type, and an appropriate buffer must be used to prevent, for example, post-sorting rupture of cell membranes. For examples of protocols for different pollen structures see Reference 9. As with all analyses involving gating, a critical step in presenting results is to describe the gating process in a clear and reproducible way.

2.2 | Nuclear DNA content: Genome size and cytotype

The use of FCM to measure genome size, whether for its own sake or as a step in determining ploidy, is arguably the most common application of FCM in plant sciences. Although to date it has been rarely done, genome size measurements can be done accurately using pollen nuclei [4], potentially providing opportunities for the collection of an alternative tissue in the field. Once a 1C DNA content is obtained, it may be used to assign a cytotype to the plant, and all the usual considerations with ploidy assignment apply [34]. But while pollen and spores may simply be alternative tissues for genome size studies, other novel uses have been made specifically of pollen FCM, including verifying the identity of the 2C peak in endopolyploid somatic tissue samples by comparison to the 1C peak position in the same plant's pollen [35, 36], confirming ploidy conversion of individual flowers in colchicine-treated plants [37], and confirming meiotic mutants producing *2n* gametes [38, 39]. A recent study of Kuo et al. [20] used FCM to provide a first thorough insight into 1C DNA content variation in ferns and lycophytes. The FCM analysis of spores is in many aspects similar to pollen FCM and can be also used for assessing the mode of reproduction. Approximately 10% of pteridophytes are apomictic and produce unreduced spores (i.e., diplospores). The apomictic species can easily be discriminated from sexuals by comparing the DNA content of spores with that of sporophytic tissue (usually leaves).

In addition to estimation of genome size based on mean fluorescence measures, variation in the fluorescence of 1*n* nuclei can provide information about aneuploidy or reveal the presence of B-chromosomes (i.e., accessory chromosomes outside the normal karyotype). In species

with higher chromosome counts, aneuploidy may be detectable based on higher coefficients of variation (CVs) of the nuclei peaks relative to euploids [13]. In species with B-chromosomes, these are often distributed unequally across cells during meiosis and mitosis, resulting in variations in chromosome number and DNA content both among and within pollen grains produced by the same plant. Wu et al. [14] showed that in *Aegilops* plants with B-chromosomes, multiple 1*n* DNA contents could be detected. Kron, Eisen, Caruso, and Husband (unpublished; Figure 5) have also demonstrated variation in DNA content of both vegetative and generative nuclei in *Lobelia* plants with different B-chromosome numbers. This method is only useful in species with relatively low chromosome numbers as DNA content differences due to single chromosomes must be distinguishable.

General considerations for measuring DNA content, such as the histogram quality standards and internal DNA content standardization, are the same as for other tissues. Some considerations are specific to pollen, however. A method for combining pollen and somatic nuclei in a sample, one as the test tissue and the other as an internal standard, is described in Kron and Husband [4]. In binucleate species, 1C (vegetative) and 2C (generative) nuclei are present, and because vegetative nuclei may be underrepresented, the 2C peak may be misidentified as the 1C peak in a sample with high debris levels [4]. In addition, very high proportions (up to 100%) of unreduced (2*n*) gametes may be produced in some plants, such as plants with odd ploidies [4] or meiotic mutants [38], and nuclei from these might also be mistaken for 1*n* nuclei.

PI is generally recommended over DAPI for genome size studies because it is not base-pair specific [40]. However, where highly accurate genome size estimates are not required (e.g., ploidy determination), DAPI may have some advantages for pollen analyses. DAPI may be more efficient because it also stains nuclei still within sperm cells but may be less useful for distinguishing vegetative and sperm nuclei (Section 1.2.2). When considering DAPI as a stain, however, be aware that if the light source is not a laser (e.g., a lamp or LED chip), parameters critical in many pollen applications may not be available, for example, SSC and FSC commonly used in debris gating (Figure 1). With respect to dye choice in general, autofluorescence of sporopollenin-containing pollen wall fragments in samples may contribute dramatically to background debris in a species-specific way, and it is likely that this autofluorescence may have less spectral overlap with some dyes than with others. For sporopollenin emission values see [41].

2.3 | Pollen development and male gamete formation

The measurement of cell and nuclei traits by FCM has helped shed light on aspects of pollen development and male gamete formation (i.e., microsporogenesis and microgametogenesis). Scatter characteristics of FCM-assessed microspores change during their development, and the sample-wide average of scatter traits can be used as an indicator of developmental stage [42]. Lo and Pauls [42] used this to demonstrate that temperature affects the rate of microsporogenesis. Hirano and Hoshino [3] analyzed male gamete behavior in pollen using FCM, detecting the time of formation of sperm cells and MGUs. Observation of changes in nuclear DNA content of sperm nuclei during pollen tube growth [43] is another potential application. It has also been proposed that impedance FCM can be used to discriminate developmental stages in pollen [44]. After crossing three *Arabidopsis thaliana* fluorescence-tagged lines (FTLs) expressing different fluorescent proteins in their pollen, Yelina et al. [45] used FCM for the intensive screening of crossover rates between homologous chromosomes during meiosis. Taking into account the relatively low number of crossovers per chromosome, this method allowed gaining much more robust estimates of crossover frequency than conventional approaches.

2.4 | Unreduced (2*n*) gametes

The estimation of proportions of unreduced (2*n*) gametes was one of the first proposed applications for pollen FCM [46], although rigorous descriptions of methodology have only appeared in recent years [4, 11]. Recently, there has been an increased interest in estimating rates

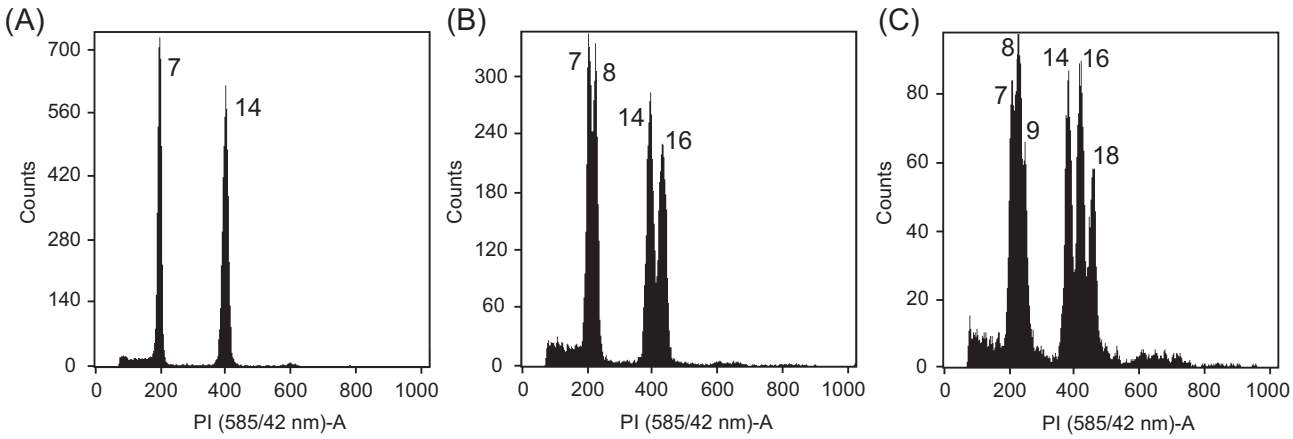

FIGURE 5 Vegetative and generative nuclei peaks in pollen from *Lobelia siphilitica* plants with (A) no B-chromosomes (2n = 14), (B) one B-chromosome (2n = 15), and (C) two B-chromosomes (2n = 16). 2n values refer to the somatic chromosome number of the plant. Numbers on the histograms indicate the chromatid numbers of the 1C vegetative nuclei (7–9) and of the 2C generative nuclei (14–18)

of 2*n* gamete production in plants using FCM, and some potentially promising applications include the experimental study of genetic and environmental drivers of gametic non-reduction [16], screening of natural plant populations for variation in rates of 2*n* gametes [10, 15], and assessing the relative contribution of 2*n* male gametes to the overall complexity of reproductive interactions in taxonomically intriguing plant groups, for example, facultative apomicts, mixed-ploidy systems; [17, 47].

Estimates of 2*n* gamete production using whole pollen grains should be considered cautiously, as whole pollen fluorescence, including autofluorescence of the pollen wall, does not produce clearly distinguished 1C and 2C clusters [48]. A more robust approach is to use nuclei extracted from pollen. At its simplest, the approach is to count nuclei from reduced (1*n*) and unreduced (2*n*) gametes (1C and 2C nuclei in trinucleate species, 1C, 2C, and 4C generative nuclei in binucleate species) and to calculate from these the proportion of 2*n* pollen grains. In trinucleate species, this is simply the number of 2C divided by the total nuclei; for binucleate species with 2C generative nuclei at maturity, the formula should incorporate 1C, 2C, and 4C events (see formula details in Reference 11).

In reality, the process of counting unreduced gamete nuclei is complicated by difficulties with gating the 1C and 2C event populations, and with excluding non-target particles from the 2C gate. Gating difficulties result because pollen nuclei peaks often deviate from normality, exhibiting a shoulder on the right side of the peak when viewed as a fluorescence histogram; when viewed on a biparametric scatterplot (e.g., fluorescence vs. SSC) this shoulder can be seen to be composed of events with both higher SSC and fluorescence, forming an upward and right-pointing tail on nuclei clusters (Figure 3). This phenomenon is likely due to either differential staining of vegetative and sperm nuclei [22] (Section 1.2.2) or to the presence of MGUs consisting of a (stained and fluorescing) vegetative nucleus attached to (unstained) sperm cells. This can make the gating of nuclei clusters difficult. This phenomenon is probably less of a problem with DAPI staining, in which differential staining (vegetative vs. sperm, free nucleus vs. cell) may be less pronounced. Further research into this phenomenon is required.

In pollen samples, the peaks of interest in estimating 2*n* gamete production will almost certainly include events other than single gametic nuclei, including doublets (aggregates), debris, and contaminating somatic tissue nuclei [11]. Estimates of 2*n* gamete production that do not address this issue should be considered highly suspect, as Kron and Husband [11] demonstrated that 2C events in trinucleate species may consist of more than 80% doublets. High doublet counts may be the result of MGU formation, for example, pairs of sperm cells in trinucleate species retaining the MGU bond and fluorescing in the 2C range [3, 11, 29]. Assuming that MGUs account for most doublets, the presence of doublets is likely to be less problematic in binucleate species, where 3C MGUs will not overlap the peaks of interest (1C, 2C, and 4C).

Doublet correction with pulse analysis (Section 1.2.4; Figure 4) works in some species, but in many it does not because of nuclear morphology [11]. Aggregate correction using histogram curve-fitting algorithms is problematic because it assumes that all particles are equally likely to form aggregates, which is unlikely to be true in pollen due to MGUs [11]. In many cases, accounting for doublets may be restricted to adopting doublet-reduction approaches (e.g., syringing samples). Somatic tissue contamination should be controlled by using pollen samples that are as pure as possible, and some methods of nuclei extraction (e.g., filter bursting, osmotic bursting) are likely to release fewer somatic nuclei than others (e.g., chopping). Nevertheless, even with careful sample preparation, somatic nuclei may contaminate samples, a serious concern considering that the events of interest (2*n* gamete nuclei) are typically present only at low levels (<1%) [10]. If contaminating somatic tissue is endopolyploid, a post-measurement analytical correction for somatic nuclei can be made based on counts of 4C nuclei in the sample and estimates of 2C/4C ratios in the somatic tissue [16]. Regardless of how doublets and somatic contamination are addressed, discussion of the method is critical in the presentation of results. It is worth noting that while doublets and somatic nuclei may influence estimates of rare 2*n* events, the results of statistical comparison of experimental treatments are not necessarily affected by correction for their presence [16].

Finally, it should be noted that estimation of 2*n* gamete frequencies is an example of an application that relies on the accurate and precise estimation of proportions, and statistical considerations related to such estimates apply, as discussed in the next section. As an example of how sample size can impact estimates of 2*n* frequency, low overall nuclei counts may give the false impression that they are absent ("2*n* non-producers") when in fact they are only rare (rare "2*n* producers") [10]. For example, for a plant producing 0.5% 2*n* gametes, if only 500 nuclei are measured, the expected number of nuclei from 2*n* gametes is 2 or 3, an event count that could easily be overlooked or lost in the debris. In fact, the probability of detecting zero nuclei from 2*n* gametes at this sample size and 2*n* frequency is $(1–0.005)^{500} \times 100\% = 8.2\%$. Fortunately, one of the strengths of FCM is the ability to rapidly measure large numbers of particles, reducing the probability that plants will be labeled as "non-producers" due to inadequate sample sizes [10]. To conclude, estimates of 2*n* gamete frequencies should always be considered in the context of sample sizes within individuals (nuclei number) and across individuals (plant number) as well as of the objectives of the study.

2.5 | Counts and proportions of particle types

Simply counting one particle type (e.g., pollen or spore number) is sometimes the goal of a study [49]. Far more commonly, studies involve estimates of proportions of two or more particle types. Aside from the estimation of 2*n* gamete frequencies (Section 2.4), we discuss a number of less common variants of this approach below. All have specific methodological considerations best addressed by the literature cited. Overall, it should be remembered that whenever proportions are estimated, the sample size will be a consideration in determining error. FCM frequently provides higher sample sizes than alternative approaches, but it is still important to develop protocols

that maximize event counts while maintaining the integrity of the particle discrimination steps.

2.5.1 | Vegetative, generative, and sperm nuclei

As described in Section 1.2.2, relative counts of intact sperm cells, free sperm nuclei, and vegetative nuclei can be made using membrane-permeable and impermeable DNA stains. By discriminating between vegetative and sperm nuclei, and between DNA contents within these types, Wu et al. [14] were able to show that B-chromosomes accumulate differently in vegetative and sperm nuclei. Discrimination of nuclei types and between cells and free nuclei could be used to assess different pollen disruption protocols: for example, the recovery rate of sperm cells versus sperm nuclei with different methods [22, 50] or the rate at which vegetative nuclei survive extraction procedures, perhaps shedding light on their under-representation in some FCM samples.

2.5.2 | Sex ratios

In dioecious plant species with heteromorphic sex chromosomes, differences in DNA content may not only allow field discrimination of male and female individuals using their FCM profiles, but maybe also used for assessing "sex ratios" in pollen they produce. Owing to this possibility, populations of two species of *Rumex* exhibiting female-biased sex ratios, *R. nivalis* and *R. acetosa* [51, 52], were analyzed in detail to ascertain whether the female bias occurs already during microgametogenesis. It should be noted that the plant genus *Rumex* is especially suitable for such studies due to its low chromosome number, and this technique may not be easily applied in other dioecious plant groups.

2.5.3 | Viable and inviable pollen

FCM has been used to estimate proportions of viable pollen in a sample. Luria, Rutley, Lazar, Harper, and Miller [53] distinguished high and low fluorescence pollen grains following staining with dichlorodihydrofluorescein diacetate, which fluoresces in the presence of reactive oxygen species, indicators of metabolic activity. After flow sorting, they demonstrated that high fluorescence pollen had good viability while low fluorescence had poor viability. This method provides a means to rapidly obtain viability estimates with high sample numbers. Alternatively, a combination of membrane-permeable and impermeable DNA stains (e.g., Hoechst 33342 and PI, respectively) can be used to separate apoptotic pollen cells from normal ones [54], serving as a proxy of pollen viability.

Sorting for viability has also been demonstrated based on pollen electric properties when exposed to radio frequencies of 0.5–12.0 MHz, using impedance FCM [44]. Though allowing rapid assessments across thousands of pollen grains, impedance FCM was also reported to overestimate pollen viability rates when compared to direct in vitro pollen germination tests [55], suggesting that the main strength of this approach could be in extensive pollen fertility screening across numerous individuals (e.g., in agriculture or breeding practice).

2.5.4 | Pollen-based taxa identification

Differences in nuclear DNA content, nuclear morphology, and whole pollen traits provide a means to distinguish between some taxa in mixed pollen samples. For example, nuclei of different estimated DNA contents were compared to a reference library of nearby species to identify source species from mixed pollen samples collected in the field from foraging honeybees and bumblebees [56]. In a similar study, pollen load composition was measured on bees foraging in a mixed diploid-tetraploid population [57]. Moon et al. [27] suggested using fluorescent protein-tagged lines (FTLs) expressing GFP in their pollen grains, distinguishing pollen from genetically modified and unmodified sources, for rapid and efficient identification of transgene flow. Another approach employs species-specific differences in structure and autofluorescence of the pollen wall [32] for automatic identification, assignment to species, and counting of airborne pollen samples, for example, see Reference 24.

Studies of this kind are limited by the extent to which the measured trait, such as DNA content or autofluorescence, can distinguish between potential candidate taxa. However, recent developments in instrumentation, including multi-directional imaging, airflow cytometry, and impedance cytometry, as well as analytical techniques such as 3D image analysis and neural networks, promise to expand this application [58, 59]. In addition, FCM data could prove to be a valuable complement to genetic methods for the identification of species, for example, pollen barcoding [60], that provide information about species composition but not abundance.

3 | GENERAL RECOMMENDATIONS

Many recommendations for pollen FCM studies are simply reiterations of those for more general studies: for example, pollen genome size studies require the same attention to sample quality and standardization as those with other tissues, and measures based on proportions have the same sample size concerns as in any other study. However, some recurring and notable issues related to pollen are summarized here:

1. Many of the methodological approaches described above have components that are species-dependent as well as application-dependent. Any pollen FCM study will necessarily require careful protocol optimization, keeping in mind the issues we have raised. In particular, alternative methods for nuclei extraction should be

tested, taking into account the quality of the output as well as ease of use.

2. Awareness of pollen diameter, obtained by microscopy or a particle counter, is critical in protocol development. Aside from concerns related to filtering out contaminating tissue, some pollen grains can be quite large (>200 μm) and can potentially cause instrument blockages, especially when clumping occurs. Choice of appropriate buffers, such as ones with detergent to reduce clumping, are required.
3. Pollen exine can be autofluorescent and so, depending on the available lasers and optics, whole pollen grain measurements beyond gross morphological ones may be difficult and may require combining FCM with other techniques.
4. Depending on the purpose of the study, previous knowledge of the taxa being studied may be critical (e.g., production of binucleate or trinucleate pollen), and attention must always be paid to the developmental stage and the likelihood that 1*n* and 2*n* microspores will be present.
5. The presence of aggregates and/or contaminating somatic nuclei cannot be ignored when the purpose of the study is to distinguish nuclei types based on DNA content. Explanations of how these particle types are managed should be considered an important part of the description of methods.
6. In many types of studies, notably unreduced gamete studies, rare event considerations are important and sample sizes (e.g., nuclei counts) are particularly relevant.
7. As in any scientific study, all the methodological steps and analytic approaches (e.g., gating procedures) should be clearly described, preferably with graphical examples.

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