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# The Rising World of Flow Cytometric Analysis of Pollen Grains

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Flow cytometry is nowadays recognized as a vital tool in plant sciences, with more and more applications in both basic and applied research (1). Due to the unique characteristics of plant material, i.e., complex three-dimensional tissues with the presence of rigid cell walls, specific methods had to be developed to produce suspensions of single particles of interest (2). These particles of interest include, nuclei, mitochondria, chloroplasts, and chromosomes, with most of the applications being focused around the estimation of nuclear DNA content (either in absolute or relative amounts), leading to increasing impacts in the fields of plant breeding and population biology.

One of the few single particles produced by plants is the pollen grain. Still, most works focused on studying pollen grains have involved the extraction of nuclei or sperm cells, rather than using the intact pollen. This is mostly due to the autofluorescence and/or specific staining of the pollen exine, and to the particle size limitations of some flow cytometers (3). To add to this, isolation of high-quality nuclei from pollen grains is often difficult, at least in comparison with somatic tissues. Together, these difficulties have led to a very minute number of publications involving flow cytometric analysis of pollen grains in the last 25 years.

Despite this deficit of studies, four different types of applications using pollen grains have been explored: (1) pollen developmental studies, including evaluation of the nuclear replication stages in mature pollen (4–6) and nuclei development in pollen tubes (7,8); (2) evaluation of the production of different nuclei types (e.g., male- and female-determining pollen in plants having heteromorphic sex chromosomes) (9,10); (3) measurements of pollen DNA to detect unreduced (2*N*) pollen grains, particularly explored in the field of plant breeding (11–13); and, more recently, (4) estimation of the genome size of plants (14,15).

The recent studies of Kron et al. (14,15) are of utmost relevance to advancements in this area, as they provided improved methodologies and technical approaches toward the use of pollen grains for genome size studies and for the estimation of unreduced gametes. Kron and Husband (14) presented a simple and relatively novel method for extracting pollen nuclei, i.e., the bursting of pollen through a nylon mesh. The authors compared the efficiency of this new approach with some of the methods previously used to isolate nuclei from pollen grains. The filter bursting method was evaluated in a huge dataset comprising 80 species (from 64 genera and 33 families), and yielded better quality histograms, with consistently higher yields of nuclei, than chopping, freeze-chopping, and sonication. This high success rate opens the door for the future use of pollen for estimating genome size in plants. Later, in an ingenious approach, the authors used this protocol to explore the possibility of obtaining information about pollen load composition and foraging behavior based on DNA content, i.e., to test whether pollen loads from single bees could be classified into types based on the genome size of pollen grains, and whether good estimates of proportions of different types could be made (15). Although, the information provided was affected by the complexity of the pollination environments, this approach is promising and constitutes a new tool for examining pollinator behavior and pollen transfer between species or between cytotypes.

An application that could greatly benefit from the method of Kron and Husband (14) is the study of unreduced

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pollen production by flow cytometry. However, these authors indicated that there were still technical challenges related to the estimation of unreduced pollen grains, in particular, the difficulty to distinguish unreduced nuclei (2*N* nuclei) from other particles with similar fluorescence characteristics, such as 1*N* doublets. In this issue of *Cytometry Part A*, Kron and Husband (page 943) explore a very inventive way to address this issue. The authors explored the possibility to apply pulse analysis for doublet correction, as is done already in other flow cytometric applications. As usual, the authors were methodologically very sound and precise and were able to develop a consistent and repeatable pulse analysis protocol, with the establishment of criteria for when the method could be confidently applied for doublet discrimination. The approach involved evaluation of the effect of gating in terms of the relative amount of error after and before gating (*I*), of the completeness of the gate ($p_{exc}$), and of the cleanness of the gate ($p_{dbt}$). With this in mind, the authors defined a set of criteria that needed to be met before considering pulse analysis: accuracy, at a minimum, could not decrease after the gating was applied; pulse analysis doublet discrimination could only be used if it is possible to, at least qualitatively, estimate *I* by estimating both $p_{dbt}$ and $p_{exc}$; and finally, gate placement needs to be repeatable, minimizing or completely removing subjectivity in gate placement. The authors also described the variation in fluorescence properties of pollen nuclei in three of the major plant families (Asteraceae, Brassicaceae and Poaceae), evaluating the applicability of the protocol to different types of pollen grains. All of this was made in comparison with estimates of unreduced gametes obtained visually using microscopy.

Despite only in Brassicaceae, nucleus fluorescence height and/or width in the unreduced gametes (2*N*) region exhibited bimodality, as a reflection of singlets and doublets (when compared with the unimodal distributions of the same parameters in reduced (1*N*) gametes), this approach constitutes a great improvement on the ability to obtain precise estimations of unreduced male gametes. It was further shown that pulse analysis estimates of doublet proportions were well correlated with estimates obtained with microscopy. In conclusion, this study showed, for the first time, that controlling for pollen grain doublets is very important. The obtained results can also imply that former studies focused in estimating the frequency of unreduced gametes may have overestimated the number of 2*N* singlets.

Although the analysis of pollen grains through flow cytometry is possible with a high precision due to recent advancements (at least in most cases), with potential significant impacts in crop and horticultural science, genome size research, and population and evolutionary biology, there are still some seldom explored applications that could increase the potential of flow cytometry for analyzing pollen grains. In the area of Ecology, in particular in studies of reproductive biology, it is very important to obtain reliable estimates of the number of pollen grains, as a measure of male fitness. Although, flow cytometry can be considered a highly sophisticated particle counter, there are only a few examples in the literature that have used this method to quantify the number of pollen grains produced in an anther, the pollen remaining in the anthers after a pollinator visit or even pollen loads over the stigmas (16), with traditional methods, such as counting pollen grains in a microscopic slide (or in a Neubauer chamber), or in a particle counter, still largely predominating. Although the use of flow cytometry is limited by the size of the pollen grain, in theory it is now possible to employ some reliable methods to make absolute counting of pollen grains. These include the use of reference beads of known concentration which are added to a sample of unknown concentration, or the use of true volumetric absolute counting available in some recent instruments, which more and more reduce the probability of a counting loss for typical event rates to percentages below 2%.

Coming back to the beginning, the use of flow cytometry to the analysis of pollen grains (or pollen grain's isolated nuclei) is very promising, leading to the rise of marvelous new applications of flow cytometry and to the proliferation of novel studies.

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