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Flow Cytometry Applied in Tissue Culture

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1. Introduction

Flow cytometry is a powerful technology that allows for the simultaneous analysis of multiple attributes of cells or particles in a liquid medium. The first cytometer used was built during World War II, when [1] developed an equipment where particles flowed through the system to diffuse light through a lens, producing electrical signals sensed by a photodetector. The instrument could detect objects in the order of $\sim 0.5 \mu\text{m}$ in diameter, and is recognized as the first flow cytometer used for observation of biological cells [2]. This would be possible to identify aerosols, bacteria that would possibly biological warfare agents as well as check the efficiency of gas mask filters against particles. In 1950, the same principle was applied to the detection and enumeration of blood cells. As hematology and cellular immunology, two biological areas, that drove the development of flow cytometry [3]. Later, with improved equipment and methods, this technique was adapted to other areas of biology, including the plant kingdom [4]. Already in 1973 the German botanist Friedrich Otto Heller used the Impulszytometrie (pulse cytophotometry in German). This scientist did not imagine that it has launched a new field of scientific research, which would later be called flow cytometry in plants.

In reference to [5] that developed a rapid and convenient method for the isolation of plant nuclei by cutting the same tissue in a lysis buffer consisting of a buffer to destroy the cellular and nuclear membranes of the cell allowing the release of DNA. Since then, this has been the main and most reliable method of isolating nuclear plant in flow cytometry. Any type of sample can be analyzed because its particles (cells, nuclei, chromosomes, cell organelles, or other cell subparticles) are suspended and vary between $0.2 \mu\text{m}$ and $50 \mu\text{m}$ in size. Solid tissues must be disaggregated and suspended before flow cytometry analysis. The suspended particles are then placed into a flow cytometry device.

The studies on flow cytometry have used as base the plant tissue culture, including the regeneration of plants subjected to chromosome doubling, for detection of somaclonal

variation in material micropropagated in various subcultures, viability of pollen grains, cell cycles and the determination of ploidy. This chapter presents results obtained through flow cytometry on plant tissue culture.

2. Preparation of material for analysis in flow cytometry

There are several methods that can be used to prepare plant material and to estimate the DNA content by flow cytometry. The methodologies differ according to plant species, a laboratory, with the brand and model flow cytometer used. In Tissue Culture Lab in the DAG / UFLA the methodology used is described in Figure 1.

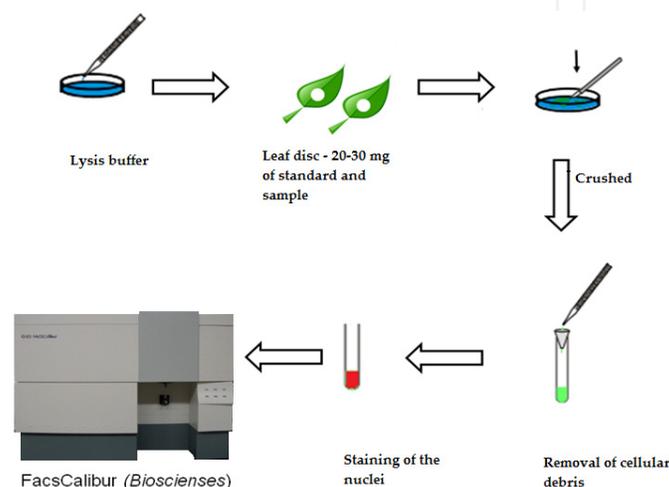


Figure 1. Diagram of the methodology used to analyze the nuclear DNA content from plant tissue. Source: Adapted from [6].

The sample must be in the form of a suspension of single particles [7] or being analyzed by flow cytometry. Analysis of DNA content by flow cytometry is based on the fluorescence intensity of nuclei stained with a fluorochrome specific to the DNA. There is a problem related with low capacity of penetration of fluorochromes but this can be overcome if the nuclei are released prior to staining. [7]. Secondary metabolites can interfere in cellular content and color of the fluorescent dye [8].

There are several methodologies developed for the release of the nuclei of plant tissues. However, the methodology proposed in reference [9] can promote the release of the nucleus (Figure 1) and is frequently used for simplicity and speed. The differences observed between the methods are the composition of the lysis buffer for isolation of nuclei, the fluorochrome for nuclear staining of the suspension and reading the sample in the flow cytometer.

Plant tissue samples are perforated, with the aid of a cutting blade, in a buffer solution for the extraction and isolation of nuclei. Subsequently, the suspension is filtered by a fine mesh nylon (20-100 μM pore diameter) [10]. This filtering is performed in order to remove all the material in the sample greater than the core, leaving in solution only those estimates and thereby obtaining the DNA content of more reliable. Furthermore, the presence of other components and soluble substances such as chloroplasts, mitochondria, phenolic

compounds, DNase, RNase etc., which are released in the cytosol may through this filter and compromise the quality of results. An alternative may be employed to remove such debris is the washing of the nuclei using centrifugation and resuspension, and to modify the components and / or pH of the buffer [11]. After filtering the samples are stained with a fluorochrome specific, then the analysis of samples in the flow cytometer.

3. Factors that affect the quality of the sample

Several factors can affect the quality of the samples and consequently the reliability of estimates of DNA content obtained by flow cytometry. Extraction buffer, reference standard, fluorochrome, type of plant tissue used (chemical composition and the presence of anthocyanin, phenolic compounds that inhibit DNA staining), quality of the sample (plant age, presence of injuries, diseases ...), storage time of the plant tissue, care in preparation and sample analysis are among the factors involved [12]. Thus, an appropriate methodology is necessary for each species.

3.1. Nuclear extraction buffer

The extraction buffer is an appropriate solution that has the function to release the nuclei of intact cells, preserving and ensuring the stability and integrity of nuclei during the experiment, inhibiting the activity of nucleases, and providing optimal conditions for staining of DNA by stoichiometry [13]. Approximately 25 caps are, but only eight are commonly used in flow cytometry [14]. The six most commonly used buffers are shown in Table 1.

Buffer	Composition	Standard
Galbraith	45 mM MgCl ₂ ; 30 mM citrato de sódio; 20 mM MOPS; 0.1% (v/v) Triton X-100; pH 7.0	[15]
LB01¹	15 mM Tris; 2 mM Na ₂ EDTA; 0.5 mM espermina.4HCl; 80 mM KCl; 20 mM NaCl; 0.1% (v/v) Triton X-100; pH 7.5	[16]
Otto's	Otto I: 100 mM ácido citric mono hidratado; 0.5% (v/v) Tween 20 (pH approx. 2–3) Otto II: 400 mM Na ₂ PO ₄ .12H ₂ O (pH approx. 8–9)	[17], [18]
Tris.MgCl₂	200 mM Tris; 4 mM MgCl ₂ .6H ₂ O; 0.5% (v/v) Triton X- 100; pH 7.5	[19]
Marie	50 mM glucose; 15 mM NaCl; 15 mM KCl; 5 mM Na ₂ EDTA; 50 mM citrate de sódio; 0.5% Tween 20, 50 mM HEPES (pH 7.2), 1% (m/v) polyvinylpyrrolidone-10 (PVP-10)	[20]

EDTA = ethylenediamine tetraacetic acid; HEPES = 4-2 ethanesulfonic Acid Hydroxyethyl-piperazine-1; MOPS = 3 - (N-morpholino) propanesulfonic; = Tris (hidroximetil) aminomethane and PVP = polyvinyl pyrrolidone.

Table 1. Composition of extraction buffers commonly used in flow cytometry plant.

The caps have in their composition organic buffering substances, non-ionic detergents and stabilizers of chromatin. The substances commonly used are buffers, MOPS, HEPES, and

TRIS, allowing the stabilization of pH 7-8 solutions, which is the pH range compatible to most of the fluorochromes used.

The nonionic detergents are present in the buffer solutions with TRITON X-100 and Tween-20, for cleaning of the cores and separation for avoiding that they add to each other or with possible debris present in the sample.

Stabilizers used in the composition of the buffer are MgCl₂, MgSO₄ and spermine and chelating agents such as EDTA and sodium citrate. These components bind divalent cations which are cofactors endonuclease. The inorganic salts NaCl and KCl allow to achieve adequate ionic strength [21].

Cytosolic compounds that are released during the isolation of nuclei, interact with nuclear DNA and / or the fluorochrome, and affect the quality of the sample and cause stoichiometric errors [22, 23, 24, 25].

In the literature there are few reports that compare the efficiency of different buffers for nuclear extraction. There is a single buffer works optimally for all types or tissues and plant species, previous studies are needed to identify the most appropriate buffer for each species studied and contribute to a greater experimental precision [24].

3.2. Reference standards fluorochromes

The reference standard is a DNA of species whose amount already previously known, and thus can be estimated by comparing the DNA content of any kind. There are a number of reference patterns with a wide range of DNA content allowing coverage of a wide range of genome. a species whose amount The use of these standards allows comparison of results obtained in different laboratories.

Estimates of DNA content obtained by flow cytometry are always relative to a standard whose DNA content is already established. This pattern receives two reference designations internal standard, when extraction of the cores and the analysis of sample and standard are performed simultaneously, or when an external standard is performed separately. The internal standards are most recommended, especially in high-precision measurements, because the peaks of the standard used and the sample appear in the same histogram and are treated under identical conditions [26] thereby reducing possible errors due to oscillation of the device during the evaluation of the samples. However [27] reported that the simultaneous processing of the sample and the reference standard was not necessary to obtain reliable estimates of DNA. It is common to use only one reference standard in all analyzes of the same experiment, but this procedure carries the risk of error due to nonlinearity [28, 29].

However, the choice and correct use of reference standards is a criterion that has been largely neglected [30].

The researcher Jaroslav Doležel from Laboratory of Molecular Cytogenetics and Cytometry, of the Czech Republic has set benchmarks with content from genomic DNA with different sizes.

Description	DNA content (pg)	References
<i>Raphanus sativus</i> cv Saxas	1,11	[31]
<i>Solanum lycopersicum</i> cv Stupické	1,96	[21]
<i>Glycine max</i>	2,5	
<i>Zea mays</i>	5,72	
<i>Pisum sativum</i> cv Ctirad	9,09	[32]
<i>Secale cereale</i>	16,19	
<i>Vicia faba</i>	26,90	
<i>Allium cepa</i>	34,89	

Table 2. Content of DNA of known standards are used.

3.3. Fluorochromes

The choice of fluorochrome is another important factor that affects the reliability of estimates of DNA content. The fluorochromes specifically bind to DNA and stoichiometrically in accordance with the intensity of fluorescence of the nucleus or the cell suspensions analyzed on flow is estimated for DNA content [6]. Fluorochromes used in coloring cores are shown in Table 3.

Fluorochrome	DNA binding mode	Wave-length	
		Excitation	Emission
Propidium iodide	Interleaving	525 (Blue-green)	605 (Red)
Ethidium bromide	Interleaving	535 (Blue-green)	602 (Red)
SYBR Green	Interleaving	488 (Blue)	522 (Green)
DAPI	Rich regions in AT	345 (UV)	460 (Blue)
Hoechst 33258	Rich regions in AT	360 (UV)	460 (Blue)
Chromomycin A3	Rich regions in GC	445 (Violet-blue)	520 (Green)
Mithramycin	Rich regions in GC	445 (Violet-blue)	575 (Green)

Table 3. Fluorochromes used in flow cytometry to estimate the DNA content.

There are two classes of fluorochromes the intercalating and specific. The propidium iodide, ethidium bromide and Sybr Green are intercalating fluorochrome, i.e., without preference of base pairs and are the most adequate to estimate the DNA content [33 cited by 34].

DAPI, Hoechst 33258, Chromomycin A3 and Mithramycin fluorochromes are specific. The Mithramycin, Chromomycin and the Olivomycins are fluorochromes which preferentially bind regions of DNA in GC-rich [35]. While the fluorochrome DAPI and Hoechst (33342 and 33258) were also specific DNA binds to AT-rich regions [36]. Therefore, the use of these dyes can lead to many incorrect estimates of the values of DNA content, since it is not known in advance the ratio of AT GC in species to be estimated the DNA content.

The propidium iodide has the lowest coefficient of variation obtained in using the fluorochrome is most suitable for determining the amount of genomic DNA in plants [37, 38, 39]. However, other authors reported propidium iodide and ethidium bromide are not dye specifically the DNA, they dye RNA too, but to not compromise the efficiency of the determination of content DNA can be used RNase [40].

4. Care use of cytometry

Below are listed some precautions that should be taken during the use of flow cytometry:

1. Avoid filling the tank of saline to their maximum capacity. When a tank is filled with pressurized fluid is forced toward the air hose preventing adequate pressurisation of the enclosure.
2. When working with propidium iodide, should be placed approximately 400 ml of hypochlorite in the sewage tank, which has a capacity of 4 liters, since the chlorine inactive molecules iodide.
3. It should be cleaned daily after use of the cytometer, the following steps: with the "RUN" button, install the probe tubes containing 3 ml of 0.5% hypochlorite, left to run on HI for 1 minute with the arm 5 minutes to open and close the arm. Select the fluid control "STNDBY." Remove the tube and insert another tube containing 1 ml rince facs (which is a detergent that helps remove waste from dyes into the machine) and let it run for 2 minutes in HI, with the arm closed. Select the button again fluid control "STNDBY" Remove everything and place another tube containing 3 ml of distilled water and let it run one minute with the open arm in HI and 5 minutes with the arm closed. Select button "STANDBY" and then install a tube containing no more than 1 ml of distilled water in the probe, because it always returns to the saline and the tube makes the volume of the tube exceeds its maximum capacity if it has more than 1 ml of distilled water, and this can affect equipment performance.
4. The tube should remain in distilled water to prevent probe salt deposits are formed in the sample injection tube
5. It should be cleaned monthly. This procedure is performed on the entire fluid system and once a month, or more often as needed. It should be removed from the reservoir containing saline solution and then install a different container with 1-2 liters of 0.5% sodium hypochlorite, flush for 30 minutes, while in the probe set 3 ml of hypochlorite solution at the same concentration . After this period must be installed to another container containing 1 to 2 liters of distilled water and left to run for 30 minutes, while the probe install a tube containing 3 ml of distilled water at the same concentration. During this procedure, iodide should never pass through the filter of saline, as you may damage it, so the hose to the filter should be disconnected during this process. Following the procedure returns the brine tank to the right place and connect the hose from the filter.
6. If the equipment becomes more than a week without being used, the salt tank must be replaced by distilled water and left to run for about 10 minutes to remove any salt of the capillary tubes of the equipment, because the salt form crystals which can clog the entire system.

7. Never replace the air tube into the sample if the button "HI" is on, the tubes should always be replaced with the "STNDBY" button and you must not allow the sample to be sucked through the probe, thus preventing air from fluid system.
8. All bubbles are displayed in the hoses from the tank and filter salt must be removed before the reading of the samples, because it makes the reading very slow. If you suspect bubbles within the system must press the "PRIME", because it injects a blast of air across the system and then complete with saline, removing bubbles. This procedure should be repeated 5 times to really solve the problem.
9. Should perform preventive maintenance on a flow cytometer, once a year by specialized professionals.

5. How to troubleshoot an analysis of flow cytometry

Paul Kron of Integrative Biology University of Guelph 10 list of solutions to problems have a histogram of quality estimates DNA content trusted. These solutions are listed below and have some adjustments based on the experiences gained at work in the Laboratory of Tissue Culture UFLA.

5.1. Verify that the flow cytometer is running well and is configured correctly

A quality control test should be performed daily and periodic maintenance by a technician from the manufacturer. These precautions ensure the proper functioning of the device.

Verify that the parameters were set by someone who is qualified to do so. Depending on the application we can use fluorescence intensity (height) or integrated fluorescence (area), linear or logarithmic scale and is vital to know the parameter most suitable for your dye.

5.2. Use good quality plant tissues

For most samples sheets are used, which should be healthy, young and cool. Sheet that shows any sign of senescence should be avoided; leaf collected at the end of the growing season often does not work. Avoid using wilted leaves.

For some species the leaves can be stored in refrigerator for 1 to 5 days after collection, since it kept in sealed plastic bag with some moist cotton. Do not leave the sample in direct contact with ice, or excessive moisture. It is also possible to store dried tissue, making use of desiccants substances. More tests are needed in this area to define protocols desiccation.

5.3. Use the appropriate tissue

If the swatch does not work, it is possible to test embryos, shoots, roots, flower petals, fruit or other healthy tissue. However, for certain species may occur the endopolyploidy, i.e. the

degree of ploidy may vary between tissues, several peaks appearing in the histogram. In this case must be used whenever the first peak to DNA content.

In case of use of seeds is necessary to attend the endosperm and embryo differ in ploidy, and the seeds may be hybrids [41].

5.4. Use the correct buffer

The choice of buffer can have a huge impact on the quality of data. This choice can influence the relative fluorescence, and the quality peak [11].

It is necessary to test not only buffers, but also the consistency of results. It is possible that a buffer can lead to production of very clean samples with low CV, but in highly variable repetition of the measures of fluorescence [42].

The pH of the buffer must be between pH 7-8.

5.5. Adjust the quantity of tissue and / or excessive cutting the sample

Excess sample is cut on a common problem and can overload the buffer, reducing their ability to maintain the correct pH range, dark coloration and large amounts of precipitation are not good signs. Keep samples on ice during cutting may help. It is possible to improve the quality of the sample cut by at least increase the amount of buffer, or by reducing the amount of tissue in the sample.

It is important to worry about getting good quality at the peaks (low CV), not number of cores. One should not impair the quality of the sample in search of "10.000 colors." This approach is often misapplied, and is more usual in analyzes of cell cycle. The core guide 1300 is the best for many applications [42]. A clean sample of 500 events per peak will probably tell you more than 10.000 events with peaks of large particles and high CV histogram very jagged.

5.6. Adjust the conditions of time and coloration.

After 2 hours of sample preparation buffer, the cores may begin to degrade. Ideally, the sample should be read in a short time after staining with 10 minutes to 2 hours, as the extreme limits.

During the stages of sample preparation, staining and reading is essential to keep them on ice and then the color should keep them in the dark, not to lose fluorescence until the moment you put them on the cytometer.

5.7. Try centrifugation

An alternative to improve the quality of the histograms is cut into a sample buffer, centrifuged (slow speed for 05-10 min), remove the supernatant and suspended again the pellet in 0.5 ml buffer, then filter and staining. This can clean up some samples.

5.8. Try a different pattern

Histogram bad when you are on a second species such as an internal standard, there may be interference between the two species of plants used (for example, by the effect of secondary metabolites) [24].

5.9. Make a gate in their samples

Even when the peaks are small and there is debris (dirt), the peaks can be measured with appropriate software making Gates. However, the removal of debris through the gate can affect how the curve fitting software analyzes of the histograms. Moreover, by making a very large suppression of scattering nuclei generates peak with a CV that both subjective and possibly artificially low, so methods of gate should be clearly described in any publication.

The samples with large amounts of debris over the cores must be considered suspect because the debris may be interfering in the coloring. Gate histograms in such poor quality must be made only when all other options fail.

Some other things to consider:

- Some tissue types may require special approaches. For example, pollen cores can be difficult to extract, as well as cutting methods and may be required for a review, see [43].
- Not all flow cytometers are equal. Some may produce better results than others, depending on factors such as size of the nuclei. If you have the opportunity to try more than one machine, the results can be enlightening.

6. Applications of plant flow cytometry

6.1. Tissue culture

Flow cytometry and microsatellite analyses were used to evaluate the trueness-to-type of somatic embryogenesis-regenerated plants from six important Spanish grapevine (*Vitis vinifera* L.) cultivars. Tetraploid plants were regenerated through somatic embryogenesis from all of the cultivars tested with the exception of 'Merenzao'. In addition, an octoploid plant was obtained in the cv. 'Albariño', and two mixoploids in 'Torronte's'. The most probable origin of these ploidy variations is somaclonal variation. The cv. 'Brancellao' presented significantly more polyploids (28.57%) than any other cultivar, but it must be noted that 50% of the adult field-grown 'Brancellao' mother plants analysed were mixoploid. Hence, it is probable that these polyploids originated either from somaclonal variation or by separation of genotypically different cell layers through somatic embryogenesis. Microsatellite analysis of somatic embryogenesis-regenerated plants showed true-totype varietal genotypes for all plants except six 'Torronte's' plants, which showed a mutant allele (231) instead of the normal one (237) at the locusVVM5. There was

not a clear relationship between the occurrence of the observed mutant regenerated plants and the callus induction media composition, the developmental stage of the inflorescences, the type of explant used for starting the cultures or the type of germination (precocious in differentiation medium or normal in germination medium) in any of the cultivars tested, except 'Torronte's' [44].

In addition, flow cytometry was used in breeding programmes to determine ploidy status after colchicine treatment of banana plants.

In reference [45] objective was to assess the colchicine and amiprophos-methyl (APM) concentration and exposure period in the chromosome duplication of banana plants diploids. Banana stem tips were used from the following genotypes: breed diploids (1304-04 [Malaccensis x Madang (*Musa acuminata* spp. Banksii)] and 8694-15 [0337-02 (Calcutta x Galeo) x SH32-63]). Colchicine was used at concentrations of 0 (control treatment), 1.25, 2.5 and 5.0 mM, while APM was used at 0 (control treatment), 40 and 80 μ M, in solution under agitation (20 rpm), for 24 and 48 h periods. With the use of APM, 66.67% tetraploid plants were obtained in the 1304-04 genotype using 40 μ M for 24 h and 18.18% in 80 μ M for 48 h, while in the 8694-15 genotype using 40 and 80 μ M colchicine for 48 h, 27.27 and 21.43% tetraploid plants were observed, respectively. For colchicine, in the 1304-04 genotype, only the 1.25 mM treatment for 48 h presented 25% tetraploid plants and in the 8694-15 genotype, the 5.0 mM concentration for 48 h produced 50% tetraploid plants. APM for 24 h enabled the tetraploid plant of the 1304-04 genotype to be obtained, while colchicine for 48 h resulted in tetraploid plants in the 8694-15 genotype.

Further, the efficiency of production of doubled haploid plants in canola (*Brassica napus* L.) breeding programmes is reduced when large numbers of haploid and infertile plants survive until flowering. Cytometry was used to assess ploidy status and predict subsequent fertility of microspore-derived plantlets from three canola genotypes, with or without colchicine treatment of microspore suspensions. Young leaf tissue was sampled from microspore-derived plantlets within 1 week of transfer to soil, and processed immediately by flow cytometry. The process was repeated on the same plants 3–5 weeks later. Of the 519 plants transferred to soil, 57.2% were consistently haploid at both sample times, 33.5% were consistently diploid at both sample times, and the remainder (9.2%) were uncertain or inconsistent in ploidy status across sampling times. Of the 518 plants that survived to flowering, 32.4% were diploid at both times of sampling and fertile (set seed) and 46.3% were haploid at both sampling times and infertile. Another 10.8% were haploid at both sampling times and fertile, but had low pollen viability and seed set, and some were triploid or of uncertain ploidy level. Colchicine treatment of microspore suspensions significantly increased the proportion of diploid plants from 9.7 to 69.7%, with significant variation among genotypes. Evidence from simple sequence repeat marker loci indicated that diploid and fertile plants from the control treatment (no colchicine) were derived from spontaneously doubled haploid gametes, rather than unreduced gametes or somatic tissue. Flow cytometry at the first sample time was very efficient in detecting diploid plants of which 94.2% were subsequently fertile [46].

We conducted a study of the cell cycle of coconut palm tissues cultured *in vitro* in order to regulate regeneration. Cell nuclei were isolated from various types of coconut palm tissues with and without *in vitro* culture. After the nuclei were stained with propidium iodide, relative fluorescence intensity was estimated by flow cytometry. Characterization of the cell cycle reinforced the hypothesis of a block in the G₀/G₁ and G₁/S phases of the coconut cells. A time-course study carried out on immature leaves revealed that this block takes place gradually, following the introduction of the material *in vitro*. Synchronization of *in vitro*-cultured leaves cells using 60 µM aphidicholin revealed an increase in the number of nuclei in the S phase after 108 h of treatment. The significance of these results is discussed in relation to the ability of coconut tissue cultured *in vitro* to divide [52].

6.2. Other applications

Cytometry can be used to assess the degree of polysomaty and endoreduplication [48], reproduction pathways [49], and cell cycle [47]. In reference [50] detected mixoploidy (variable amounts of DNA in tissue) and aneuploidy (variations in a small number of chromosomes) by flow cytometry [51].

Several protocols for measuring DNA have been developed, including bivariate analysis related to cytokeratin/DNA analysis/DNA analysis of BrdU and a synthetic nucleoside similar to thymine. These protocols are used to study the cell cycle and to obtain multiparametric measurements of cellular DNA content; they were developed in tandem with commercial software for analyzing the cell cycle [47].

7. Final considerations

Although flow cytometry significantly impacts several fields of plant research, various methodological challenges must be overcome before its potential can be fully realized.

The research group in UFLA's Department of Agriculture consistently attempts to use methodologies for analyzing nuclear DNA content in plants, which removes some technical constraints. We emphasize the importance of research, particularly in disseminating knowledge on best practices, such as standardization type, fluorochrome selection, data presentation, and quality outcome measures.

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