Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 320609, 7 pages
doi:10.1155/2010/320609

Research Article

Improved and Reproducible Flow Cytometry Methodology for Nuclei Isolation from Single Root Meristem

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Received 29 January 2010; Revised 30 March 2010; Accepted 6 May 2010

Academic Editor: João Loureiro

Root meristems have increasingly been target of cell cycle studies by flow cytometric DNA content quantification. Moreover, roots can be an alternative source of nuclear suspension when leaves become unfeasible and for chromosome analysis and sorting. In the present paper, a protocol for intact nuclei isolation from a single root meristem was developed. This proceeding was based on excision of the meristematic region using a prototypical slide, followed by short enzymatic digestion and mechanical isolation of nuclei during homogenization with a hand mixer. Such parameters were optimized for reaching better results. Satisfactory nuclei amounts were extracted and analyzed by flow cytometry, producing histograms with reduced background noise and CVs between 3.2 and 4.1%. This improved and reproducible technique was shown to be rapid, inexpensive, and simple for nuclear extraction from a single root tip, and can be adapted for other plants and purposes.

1. Introduction

Meristematic root tissue has been the object of studies ranging from chromosome analyses to cell cycle and its physiological regulations [1]. These events comprise rates of proliferating and quiescent cells, characterization of cell subsets and elucidation cell cycle length and progression, besides effects of various putative modulators and inhibitors (e.g., hormones, growth factors, and toxins) and of environmental conditions on the cell cycle, which can be screened by flow cytometry—FCM [2].

In chromosome analyses by flow sorting [3–8], root tip meristems have proved to be advantageous suppliers of metaphase chromosomes for isolation, thanks to the great readiness of cells to synchronize. Unlike *in vitro* cultures, namely, cell suspensions and leaf protoplasts, root tips represent a cheaper, easier to handle and more karyologically stable experimental system [3, 6, 9].

Considering the radicular meristem cell cycle, FCM has been considered a fast and accurate method to evaluate nuclear DNA content. This procedure allows monitoring the production of high-quality commercial seed lots after priming treatments [10]. For instance, the effect of osmopriming in pepper and tomato has been followed by FCM, employing embryonic root tips as material source [11–16].

Root meristems have also been required in other FCM applications to substitute the frequently used leaves in current protocols [17, 18]. This preference for root material from some species is based on decreased contamination of samples with plastid DNA and absence of secondary metabolites or other disturbances, which significantly reduce DNA yield and quality [19] by affecting fluorochrome accessibility to DNA [17]. In addition, the use of root tips does not involve storage length problems, especially when material collection is carried out at long distances [17, 18, 20]. In order to prove the reliability of employing radicular material, some authors have compared radicle and leaf histograms of pepper [12] and tomato [13, 14], and corroborated their 2C DNA content.

However, meristematic root tips show low yield of material per tip [21]. To overcome this limitation, different FCM procedures have been described to supply suspensions with a

large quantity of nuclei or chromosomes, although requiring a number of root meristems per sample. Chiatante et al. [21] developed a protocol for nuclei isolation, denominated gentle hand-homogenization, using a glass rod and modified devices. Sgorbati et al. [22] also employed a glass rod for the same purpose, recurrent by Sgorbati et al. [23] and Lorbiecke and Sauter ([24], with modifications). A further adopted methodology [3, 26–30] refers to the chopping technique established by Galbraith et al. [25], and other researchers used a mechanical homogenizer [1, 5]. Table 1 informs about other references that show relevant data upon implementation of FCM procedures using roots.

Considering the increasing demand for the use of roots as material source in FCM or in general studies for DNA content quantification, cell cycle analyses, seed lot evaluation, cytogenetic study, comet assay applications, and the difficulty in obtaining adequate nuclei amounts from radicular material, we describe here an improved and reproducible protocol for nuclei isolation from a single root meristem, using *Allium cepa* as a model.

2. Material and Methods

2.1. Plant Material. The species *Allium cepa* L. cv. Alice, seeds of which were kindly supplied by Dr. Jaroslav Doležel (Experimental Institute of Botany, Czech Republic), was used as a model. The procedures and analyses were carried out at the Cytogenetic and Cytometry Laboratory, General Biology Department, UFV, Brazil.

2.2. Rooting and Fixation. Onion bulbs were placed in contact with water for root induction at room temperature. After reaching 1.5–2.0 cm of length, primary roots were fixed with a methanol (Merck, http://www.merck-chemicals.com/, product number 1060091000): acetic acid glacial (Merck, product number 1000631000) solution 3 : 1 (v/v) and stored at $-20^{\circ}C$ until processing for flow cytometric analysis.

2.3. Excision of the Meristematic Region. In order to obtain as much as possible of the meristematic region alone, the following strategy was adopted. Firstly, we have developed a homemade slide as helper for cutting out the root tips. On this prototype slide (Figure 1(a)), one onion root was placed in the longitudinal fissure and the meristem, visually identifiable by its whitish color, was transversely excised in its upper extremity (between the meristematic region and the elongation zone) and in its lower end (between the meristematic region and the root cap), using a brand new razor blade, under stereoscopic microscope. This simple device allowed immobilization of the root as well as perpendicular and precise cuts, in an uninterrupted course, at the meristem boundary.

2.4. Nuclei Extraction. To remove fixative solution, the meristems were washed in distilled water three times of 10 minutes each. Next, they were softened with a pectinase solution (Sigma, http://www.sigmaaldrich.com/sigma-aldrich/home.html, product number P2736) diluted in distilled water in proportion 1 : 10 (enzyme: water) at $33^{\circ}C$ for 30 minutes. The meristems were subsequently washed again.

A single meristem was carefully transferred to a 2-mL microtube (Axygen, http://www.axygen.com/, product number MCT-200, Figure 1(b)) containing 300 μL of 4′,6-diamidino-2-phenylindole (DAPI) buffer (Partec GmbH, http://www.partec.com/cms/front_content.php, product name CyStain UV Ploidy, code number 05-5004). Nuclei suspensions were obtained with use of a commercially available mini hand mixer (Figure 1(c)) by homogenizing the solution with 2, 4, 6, 8, or 10 pulses (one pulse corresponds to approximately one second), in vertical movements, being careful to avoid twisting movements. After homogenization, the mixer was washed with 300 μL of the same buffer. Nuclear suspensions were sieved through 30 μm mesh nylon filter (Partec GmbH, order number 04-0042-2316) into cytometer reading tubes (Partec GmbH, code number 04-2000). Additional 600 μL of the buffer were dropped to wash the 2-mL microtubes, and the suspension was then filtered again.

2.5. Flow Cytometric Analysis. After 15–20 minutes in the dark at room temperature, the nuclear suspensions were analyzed with a PAS-III flow cytometer (Partec GmbH, code number 15-01-1000), equipped with a UV lamp emitting at 378 nm, excitation filters (KG 1, BG 38 and UG 1), a GG 435 long-pass barrier filter for blue fluorescence, and a TK 420 nm dichroic mirror to provide epi-illumination. The equipment was carefully calibrated and aligned using microbeads and standard solutions according to the manufacturer's recommendations. FlowMax software (Partec GmbH) was used to process the data. The instrument gain was adjusted in such way that the G_1 peak was positioned on channel 200 of the histogram of relative DNA content.

Three repetitions were carried out for each homogenization test. Assuming the best results, based on lowest CVs, ten samples were processed and analyzed each day, for two days, totalizing twenty samples, and the experimental procedure was executed by different researchers.

2.6. Cytological Analysis. Through cytological analysis we evaluated the nuclear quality (in regard to shape, integrity and isolation) after extraction procedures with 2-, 4- and 6-pulse homogenization.

Nuclei suspensions were transferred to 2-mL microcentrifuge tubes (Eppendorf, http://www.eppendorf.com/int/?l=1&action=start, order number 022431048) and centrifuged at 100 $\times$ g for 5 minutes. After discarding the supernatant, the pellets were stored at $-20^{\circ}C$ for 15 minutes in 2 mL of methanol (Merck): acetic acid glacial (Merck) solution 3 : 1 (v/v). The fixed materials were centrifuged again and the pellets were resuspended in 100 μL of fixative solution by brief vortexing. Two drops of this nuclei suspension were dripped onto a very clean slide. The slide was immediately air-dried by fast wave movements, stained with a solution of 0.5 μg/mL DAPI in PBS buffer (pH 7.4), covered with a cover slip (25 $\times$ 25 mm, Corning,

Table 1: Data of nuclear FCM analysis from root meristem according to methodology employed.

Meristem source	Root tip length (mm)	Root tip/sample	CV (%)	Nuclei/sample	Species	References
Method-gentle hand-homogenization						
Root	0–2	100 (unclear)	4.4 and 5.8	5×10^6–1×10^7	*Pisum sativum*	[21]
Method-glass rod						
Root	0–2	10	2.6 (only control)	—	*Pisum sativum*	[23]
Root	—	—	—	$\geq 1 \times 10^4$	*Oryza sativa*	[24]
Method-chopping						
Radicle	1	5–10	—	821–3852	*Solanum lycopersicum*	[11]
Radicle	1	≥20	—	$\geq 1 \times 10^4$	*Capsicum annuum*	[12]
Radicle	—	5	—	—	*Solanum lycopersicum*	[13]
Radicle	—	70–80	—	$\sim 6 \times 10^3$	*Solanum lycopersicum*	[14]
Radicle	1	5	—	5×10^3–1×10^4	*Solanum lycopersicum*	[15]
Root	—	10–30	—	1×10^4 (unclear)	*Arabidopsis thaliana*	[26]
Root	—	—	—	—	*Solanum tuberosum*	[27]
Root	~ 2	9	—	8×10^3–1×10^4	*Allium cepa*	[28]
Root	2–3	20		8×10^3–1×10^4	*Secale cereale*	[29]
Root	2–3	20	—	8×10^3–1×10^4	*Triticum aestivum*	[29]
Root	2–3	7		8×10^3–1×10^4	*Vicia faba*	[29]
Root	~2	9	—	8×10^3–1×10^4	*Allium cepa*	[30]
Radicle	1	3–5	—	3469, 4425, 7547, 18874	various	[31]
Radicle	—	15	—	1×10^4	*Hordeum vulgare*	[32]
Method-mechanical homogenizer						
Root	1–2	—	—	—	Various	[1]
Root	1	1 (and >1)	—	1×10^5/mL	*Pisum sativum*	[5]
Root	0.5–1	50	—	$\sim 1.5 \times 10^5$	Various	[19]
Root	2	—	—	—	*Vicia faba*	[33]

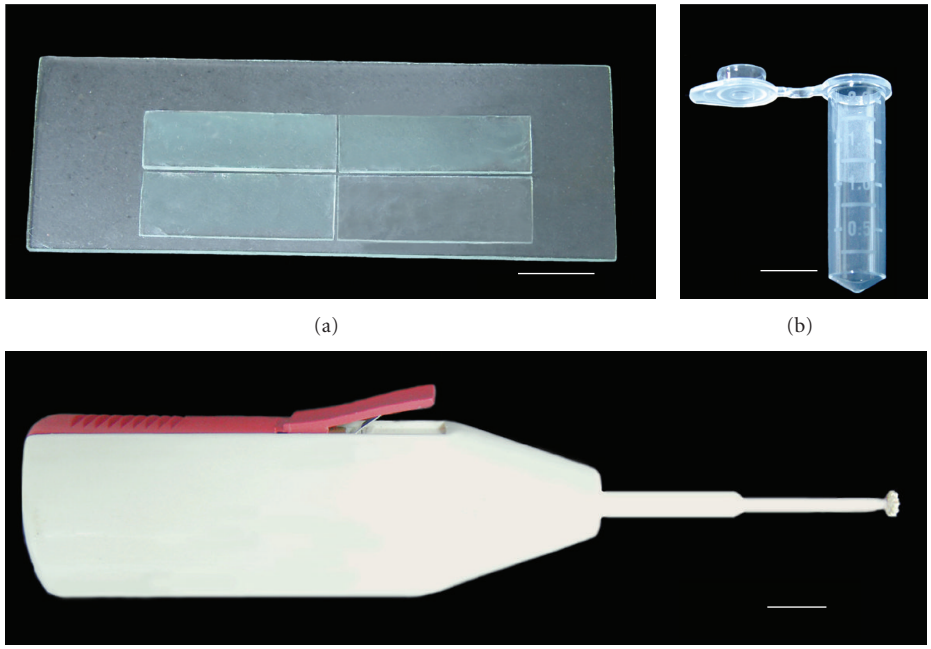
CV: coefficient of variation; —: data not shown.

http://www.corning.com/lifesciences/worldwide.aspx, product number 2865-25) and sealed with nail polish.

Images of DAPI-stained nuclei were captured with a DP71 video camera (Olympus, http://www.olympus-global.com/en/, product number D705-2), mounted on a BX-60 fluorescence microscope (Olympus), with a 100x objective lens and a WU filter (wide band cube filter: excitation wavelength 372 nm and emission wavelength 456 nm). The frame was digitized using the Image Pro-Plus 6.1 software (Media Cybernetics).

3. Results and Discussion

The use of a prototype slide (Figure 1(a)) facilitated the cutoff as much as possible of only the meristematic region of the onion root. We considered this strategy to be important for generating FCM suspensions free of nonmeristematic tissue, when the focus is the cell cycle study. This nonmeristematic tissue refers to the elongation zone, which is located just above the meristem region. Thus, the endoreduplicated cells commonly present in this zone cause the appearance of additional peak(s) on the histogram, whose interpretation can become more difficult. Some fine FCM works have mentioned a fixed length for sectioning the



(a) (b)

(c)

Figure 1: (a) Homemade prototype slide developed in our laboratory: on a brand new slide, four diamond-cut slide pieces were attached with commercial glass glue so that they could form two fissures—the longitudinal one, where a root is allocated, and the narrow transversal one, for the passage of a razor blade in a steady way. (b) Boil-proof 2-mL microtube (Axygen). (c) Mini AA battery operated hand mixer, commercially available and inexpensive (dimensions: $16 \times 3.5 \times 2.5$ cm). Bars = 1 cm.

root tip independently of the meristem size [21, 23, 29, 33]. Unfortunately, the practical manner how this was done has not been clearly described. Nevertheless, if the percentage of nuclei exclusively from meristem is considered critical, then the protocol should look into ways to delimit and extract this specific region. Such caution might be important for improving accuracy in flow cytometric analysis especially for very small material like root tips.

Preliminary tests were performed with 2-, 4-, 6-, 8-, and 10-pulse homogenization to select the best nuclei isolation, based on CVs of flow histograms as parameter of choice. The corresponding histograms are shown in Figures 2(a)–2(d), except for the 10-pulse homogenization test. The 6-pulse homogenization (Figure 2(c)) was adopted for *A. cepa*, which showed the lowest CV values. Histograms were generated from twenty individually processed roots, showing CVs that varied between 3.2 and 4.1%. The number of nuclei (G_0/G_1 peak) measured for each meristem ranged from 3108 to 7532. In fact, the histograms shown in Figure 2 only serve as a comparative guidance, since their high quality will depend on minor handling adjustments made by the executor (e.g., the way for homogenizing and pulse number) in accordance with the species studied, as happens in usual leaf chopping procedure in which some handiwork adjustments are made.

To certify nuclear quality, in regard to shape, integrity and isolation, the 2-, 4-, and 6-pulse homogenization tests were cytologically analyzed. The others (i.e., 8 and 10 pulses) were excluded due to inadequate histogram patterns, denoted by an excess of debris. In three trials, spherical shaped and intact nuclei were observed, characterizing preserved morphology. However, nuclei in clusters and surrounded by cytoplasmatic residues were formed in the first trial (2-pulse homogenization, Figure 3(a)), whereas in the second one (4-pulse) nuclei were isolated, but still evidenced cytoplasm (Figure 3(b)). As expected, the optimal 6-pulse test corroborated prior data (represented by the histogram in Figure 2(c)) inasmuch as completely extracted nuclei were visualized (Figure 3(c)).

Acid methanol fixation was considered an important step due to the promoting material storage until FCM running. Besides, this fixation process did not alter the nuclear suspensions quality. The use of this nonadditive fixative (i.e., which acts on the tissue without chemically changing it) does not interfere with quantitative staining involving DNA intercalators [20]. In the present paper, the flow cytometric protocol using DAPI (which preferentially binds to AT-rich regions) was not hampered by the fixation process.

Concerning the enzymatic digestion, this step was included to soften hard root tissue and consequently render a more gentle homogenization so as to avoid intense mechanical stress and, consequently, damaged nuclei and cellular debris. Short exposure to pectinase solution was not enough to degrade or modify neither nuclei morphology nor DNA structure. In the same way, Doležel et al. [3] discussed that longer enzymatic treatments may cause increased stickiness and even disintegration of chromosomes, in cases of flow sorting approaches.

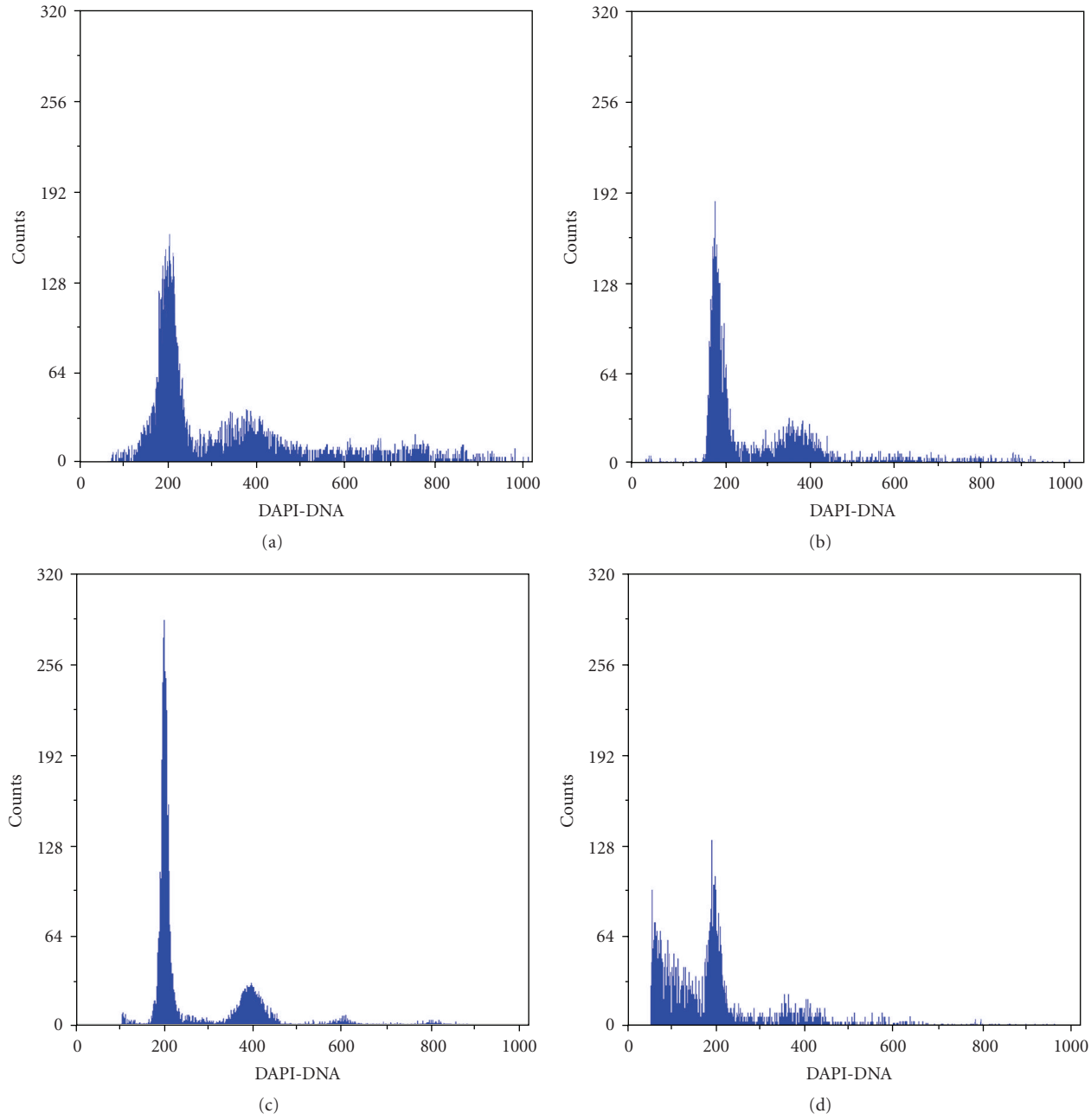
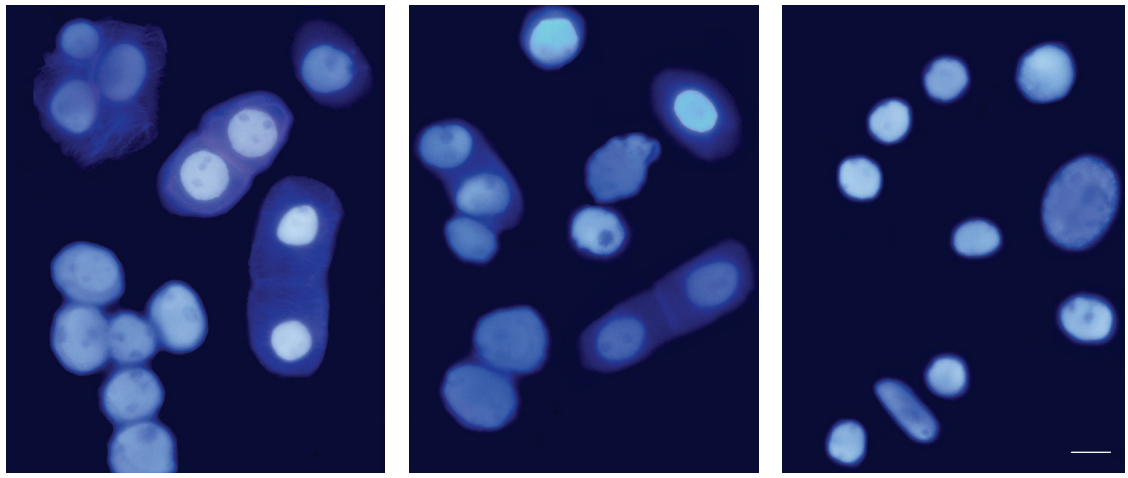


Figure 2: Representative flow histograms of DAPI-stained nuclei from *Allium cepa* root meristem after 2- (a), 4- (b), 6- (c), and 8- (d) pulse homogenization tests, showing in G_0/G_1 peaks: 4120 nuclei (CV 9.16%), 3855 nuclei (CV 7.36%), 4723 nuclei (CV 3.2%) and 2685 nuclei (CV 6.47%), respectively. Note that the most adequate histogram corresponds to 6-pulse homogenization (c).

In the present paper, use of the one-speed pulse mixer as well as the previous digestion step, facilitated the dissociation of one meristem at a time and consequently allowed the liberation of nuclei with minimal damages. Differently, Chiatante et al. [21] reported a failed attempt to obtain-high quality nuclear suspensions when they used a certain homogenizer. The mechanical stress caused by such instrument yielded nuclei with twisted shapes. But, other authors have applied the mechanical homogenizing method successfully, though processing more than one root tip per sample ([1, 5, 19]; see Table 1). Besides, many researchers have processed samples also with more than one root tip [11–15, 21, 23, 26, 29, 32] to compensate for the low quantity of isolated nuclei from the small-sized root meristem. However, the analysis of individual root meristem is fundamental in some studies, such as for investigating treatment effects or when plant number is limited.

The FCM results showed that the number of analyzed particles per root (range: 3108 to 7532) did not reach the quantity (10000) commonly found in leaf cytometric assays,



(a) (b) (c)

Figure 3: Morphological analysis of DAPI-stained nuclei, isolated from single onion root meristem after 2- (a), 4- (b) and 6- (c) pulse homogenizations. Note that after running of 6 pulses the nuclei were well preserved, isolated and showing no residual cytoplasm. Bar = 10 μm.

but they basically corresponded to DAPI-stained nuclei, as confirmed by the negligible background on the left of the G_0/G_1 peaks (Figure 2). Therefore, low CVs (below 5%), which are considered acceptable for FCM assessments [20], were achieved. These characteristics reiterate the great quality of histograms and, consequently, the high resolution of the proposed technique. Thus, it is important to emphasize these parameters in order to demonstrate the reliability of the cytometric procedure applied. Unfortunately, only few authors have regarded the inclusion of these data and CV values (Table 1), and among those, only Sgorbati et al. [23] assessed a CV value lower than the one found in this study. A possible explanation for the difficulty in obtaining low CVs from roots refers to the heterogeneity of cell cycle length in different tissue domains, belonging to derivative region immediately proximal to the apical meristem [1].

Some procedures from this new flow cytometric methodology, particularly digestion time and homogenization pulses, were specifically suitable for *A. cepa*. Nonetheless, these steps can be adjusted to other species. In our laboratory routine, results showing same quality have been obtained for other species, like eucalyptus, soybean and maize. Moreover, considering the applicability of this work for estimation of nuclear DNA content and cell cycle analyses in roots, additional aspects may also be adapted for other purposes, such as obtaining chromosome suspensions for flow sorting and radicle studies. Apart from use in FCM approaches, obtaining individualized nuclei suspensions could also be applied to the comet assay technique (single-cell gel electrophoresis), one of the most used assays for the assessment of DNA damage repair [34].

We conclude that our FCM protocol was reproducible and efficient to provide adequate nuclear suspensions for this approach from a single root meristem. Besides, the simplicity, rapidness and low cost of the described technique should not be overlooked.

Acknowledgments

The authors thank Conselho Nacional de Pesquisa (CNPq, Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil), and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, Brazil) for providing financial support for this paper.

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