Analysis of Nuclear DNA Content and Ploidy in Higher Plants

Flow cytometry is increasingly employed as the method of choice for determination of nuclear DNA content and ploidy level in plants. It thereby supplants previous methods that involved either fluorescence and absorption microspectrophotometry or chromosome counting, primarily because flow cytometry provides exceptional rapidity, convenience, and accuracy. Methods for flow cytometric measurement of DNA content have been developed for individual plant cells, protoplasts, and intact plant tissues. These methods can be employed with all commercial flow cytometers, including portable models, and hence can expect to find further applications in field biology, ecology and taxonomy, and agriculture, as well as in the systematic classification of plant-genome sizes (Galbraith et al., 1983; Galbraith, 1990; Arumuganathan and Earle, 1991; Marie and Brown, 1993; Bharathan et al., 1994; Bennett and Leitch, 1995; also see Internet Resources).

A flow cytometer is equipped with a powerful source of illumination, provided either by a laser or an arc lamp. For the use of a typical laser-based flow cytometer in estimation of nuclear DNA content with the fluorochromes propidium iodide (PI) and mithramycin (MI), see Basic Protocol. Corresponding procedures are also described for an arc lamp–based cytometer (see Alternate Protocols 1 and 2). Both types of cytometer can be employed for analysis of DNA content using fluorochromes other than PI. The emission spectrum of a high-pressure mercury-arc lamp contains lines in both UV and visible wavelengths. Thus, arc lamp–based instruments are suitable for analysis of samples stained by other fluorochromes, such as mithramycin, DAPI, and Hoechst 33258. Large water-cooled lasers can be adjusted to produce UV light; however, the high capital cost of these lasers and the cost of running them represent disadvantages.

Intact plant tissues used in the protocols below should be disease- and stress-free. For leaves, it is important to choose the appropriate age. Young, rapidly-growing leaves usually give the best results. However, developmental variations in cell-cycle behavior have been noted (see for example, Galbraith et al., 1983, 1991). Leaves may be transported or sent by post wrapped in moistened paper tissue and enclosed in a plastic bag. High temperatures should be avoided during transportation.

Protocols are also included for preparation of fixed plant materials for those instances in which analysis cannot be performed immediately after collection and processing—including fixed protoplasts (see Alternate Protocol 3), formaldehyde-fixed tissues and cells (see Alternate Protocol 4), and ethanol/acetic acid–fixed intact cells (see Alternate Protocol 5). Bulk ploidy screening—e.g., for determining ploidy in large populations of plants—is covered as well (see Alternate Protocol 6). Protocols are included for preparation of chicken red blood cells for use as internal standards (see Support Protocol 1) and for instrument alignment (see Support Protocol 2).

ANALYSIS OF SOMATIC DNA CONTENT, PLOIDY, AND CELL-CYCLE STATUS OF PLANT TISSUES USING A LASER-BASED FLOW CYTOMETER

BASIC PROTOCOL

The flexibility of laser-based flow cytometers depends on the number of lasers attached to the instrument and whether these lasers are tunable or produce light of a fixed wavelength. The following protocol describes use of the simplest instrumentation configuration—a single argon laser producing either 488-nm or 457-nm light—and two different fluorochromes, propidium iodide (PI) and mithramycin (MI). Nuclei are ob-

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tained from chopped plant material or cell cultures, then stained with PI or MI and run on the flow cytometer.

The flow cytometer should be set up according to the manufacturer's instructions for analysis of PI- or MI-derived fluorescence. The steps described below for preparing the flow cytometer are for the Coulter Elite, although the instructions are in general valid for other laser-based flow cytometers that have four photomultiplier tubes.

Materials

Plant material for analysis: intact plant tissues, plant tissue culture or callus, or plant protoplasts
Internal standards: e.g., chicken red blood cells (unfixed CRBCs; see Support Protocol 1) or plants with known nuclear DNA content (see Critical Parameters)
Homogenization buffer (see recipe)
1 mg/ml propidium iodide (PI) stock solution (see recipe)
1 mg/ml RNase stock solution (see recipe)
0.1 mg/ml mithramycin (MI) stock solution (see recipe)
Appropriate sheath fluid
Fluorescent microspheres (DNACheck, Coulter)

5.5-cm plastic petri dishes
New single-edged razor blades
15-µm pore-size nylon mesh
Flow cytometer with 488 nm (PI) or 457 nm (MI) light source
0.22-µm Millipore GSWP 047 filters
Cell-cycle analysis software (optional; Phoenix Flow Systems, Verity Software, or Eric Martz at *http://www.bio.umass.edu/mcbfacs/flowcat.html*)

NOTE: All steps must be carried out on ice; it is also recommended that procedures be performed in a walk-in cold room.

Prepare and stain suspensions of nuclei

1a. *For intact plant tissue, cell cultures, or callus:* Weigh plant materials and place in plastic petri dishes. Add 0.3 ml homogenization buffer for every 100 mg fresh weight of tissue. Chop tissues using a new single-edged razor blade, to homogenize the tissues and release the nuclei. If plants with known nuclear DNA content are to be used as internal standards, chop these simultaneously in the same petri dish with the plant tissue of interest.

Plant organs should be disease- and stress-free and ideally should comprise young, growing tissues. Cell cultures must first be centrifuged at low speed (5 min at 50 × g) to remove the growth medium. The packed cells are then transferred to the petri dish using a spatula and homogenized after addition of homogenization buffer. Callus materials can be transferred directly using a spatula.

Time of homogenization and number of "chops" required are determined empirically. Intact plant tissues typically take 2 to 3 min at ~5 chops/sec. Suspension cultures take longer to homogenize, since the cells are less readily disrupted by razor blades. The process can be monitored through epifluorescence microscopy following staining with PI. For a standard Zeiss microscope equipped with a 50-W mercury arc source, PI-stained nuclei can be observed via excitation at 510 to 560 nm, beam splitting at 575 nm, and emission detection at >590 nm. For MI-stained nuclei, the corresponding settings are: excitation 400 to 450 nm, beam splitting at 490 nm, and emission detection at >510 nm. The released nuclei should appear intact and homogeneously stained.

1b. *For protoplasts:* Resuspend protoplasts in homogenization buffer at a concentration of 10^6 protoplasts/ml. Include reference protoplasts of known DNA content as needed.

Harkins et al. (1990) provide information about protoplast preparation.

The protoplasts break as a consequence of the detergent present in the homogenization buffer.

Protoplast viability is an important consideration. Protoplasts should not be employed without gradient purification and typically should be 90% to 100% viable as determined via staining with fluorescein diacetate (Harkins et al., 1990).

2. Filter nuclear suspension through 15-µm nylon mesh.

This step is critical for removing large debris, which otherwise might block the flow cell. For nuclei of plants having genome sizes much larger than tobacco (i.e., >4.8×10^9 base pairs/haploid genome or >10 pg DNA/2C nucleus), larger mesh sizes (40 to 60-µm) are appropriate.

The nylon mesh can be conveniently placed over the tip of a standard 5-ml disposable syringe and held in place using the plastic cover provided with disposable syringes, after cutting the tip off this cover to produce a ring. Alternatively, filter units can be made using ordinary 1-ml pipet tips. Cut off the tapering tip end and gently press the cut edge on a preheated hot plate until the plastic softens (since this method produces plastic fumes, work in a fume hood). Immediately press the hot edge onto the middle of a mesh square cut a bit larger than the pipet tip. Check that the seal is fully round and complete.

3a. *To stain with propidium iodide:* Add 1 mg/ml PI stock to a final concentration of 50 to 200 µg/ml and 1 mg/ml RNase stock to a final concentration of 10 µg/ml. Let sample stand 5 min prior to flow cytometric analysis.

If unfixed CRBCs (see Support Protocol 1) are to be used as internal standards, they should be mixed with the cells prior to the addition of PI (or MI, see below).

RNase must be added to avoid binding of PI to RNA. Since mithramycin binds specifically to DNA, RNase is not used in step 3b.

3b. *To stain with mithramycin:* Add 0.1 mg/ml MI stock to a final concentration of 10 µg/ml. Let the sample stand for 5 min prior to flow cytometric analysis.

The precise concentrations of PI or MI to be employed, relative to the mass of tissue homogenized, should be determined through saturation analysis (see Critical Parameters). The time of incubation prior to flow analysis should be sufficient to permit the fluorescence signal to become invariant with respect to time.

Set up and align the flow cytometer

4. Power up the computer, cytometer, and laser. Adjust laser-emission wavelength to 488 nm (for PI) or 457 nm (for MI).

For air-cooled lasers, 20-mW power output at 488 nm is sufficient. For water-cooled lasers, 100-mW output at 457 nm is employed.

5. Prepare an adequate amount of sheath fluid that closely matches the sample fluid in ionic strength and filter through an 0.22-µm Millipore GSWP 047 filter. Empty the waste tank and fill the sheath tank with the filtered sheath fluid.

The sheath fluid should have the same ionic composition as the sample fluid (in this case the homogenization buffer) but detergents should be omitted.

6. Design and load a protocol for alignment using fluorescent microspheres.

A Coulter Elite is normally equipped with four photomultiplier tubes (PMTs). In the standard filter configuration, 90° light scatter is assigned to PMT1, green (505- to 545-nm) fluorescence to PMT2, orange (555- to 595-nm) fluorescence to PMT3, and red (670- to 680-nm) fluorescence to PMT4. The flow cell has an orifice of 100-µm diameter. For PI,

the protocol should include acquisition of single-parameter histograms of forward-angle light scatter (FS), a single fluorescence channel (PMT3; peak and integral signal, 555- to 595-nm), and biparametric histograms of PMT3 fluorescence versus time. For MI, the protocol should substitute PMT2 (integral signal, 505- to 545-nm) for PMT3. Further blocking filters (BG38) can be employed to eliminate chlorophyll autofluorescence.

7. Align the cytometer using fluorescent microspheres diluted $^1/_{10}$ with deionized water. Collect uniparametric histograms of fluorescence emission (peak and integral signal) and FS at a sample flow rate of 70 particles/sec, with the FS discriminator set to 100 and all other discriminators switched off. Adjust the optics until population coefficients of variation (CVs) for pulse integral and FS are minimized (typically <2% for fluorescence and 2% to 3% for FS).

8. Rinse the calibration beads out of the sample lines with water or buffer..

   The instrument and sample are now ready for DNA content analysis (steps 9 to 14). After initial analyses, final adjustments to the beam and flow cell positions should be made so as to obtain the lowest possible CV.

Analyze the fluorochrome-stained nuclear suspensions

9. Trigger on peak fluorescence signal.

   Although triggering is conventionally done on FS, in this case the homogenate contains many more light-scattering particles than it does nuclei. If triggering is done using FS, the desired signals from the minor population of nuclei will be obscured by the large background of particles that do not correspond to nuclei.

10. Run samples, including internal standards, at a data rate of 100 to 150 events/sec.

   Higher data rates lead to peaks that have larger CVs. A minimum of 5,000 total events should be acquired; 20,000 is recommended.

   The authors recommend that the stability of fluorescence emission be monitored over time using the internal standards. This is done via biparametric analysis of fluorescence emission versus time. The DNA content of CRBCs appears to be strain- and sex-specific (Nakamura et al., 1990). It is recommended that independent measurements of CRBC DNA content be done using non-flow-based procedures (Burton, 1968) to establish this value.

   For some plant species, the presence of secondary products (e.g., phenolic compounds) can impede quantitative analysis. This is seen as an absence of discrete peaks on the DNA histograms, or as peaks having unacceptably large CVs (>5% to 7%). Various supplements to the homogenization buffer can be helpful in suppressing these interferences, including (final concentrations in buffer): 15 mM 2-mercaptoethanol (Dolezel et al., 1994), 5% (v/v) polyvinylpyrrolidone (MW 40,000; PVP 40), 10 mM ascorbic acid, or 10 mM dithiothreitol (Bharathan et al., 1994). These supplements may be added individually or in combination; the ideal combination is determined empirically.

Analyze flow cytometric data

11. Determine the channel number of the sample G_1 nuclear DNA peak and that of the internal standard.

   The peaks are usually symmetrical, so either the mode position or calculated mean can be employed.

12. Calculate the somatic (2C) DNA content of the sample according to the equation:

$$\text{sample 2C DNA content (pg DNA)} = \frac{\text{sample } G_1 \text{ peak mean}}{\text{standard } G_1 \text{ peak mean}} \times \text{standard 2C DNA content}$$

If necessary, DNA content values can be expressed in base pairs, using the following formula: 1 pg DNA = 0.965×10^9 bp (see Internet Resources).

Whereas genome sizes in base pairs are conventionally reported in terms of the haploid size (1C) of the genome, mass values for genome size are typically reported per 2C value or even per 4C value. Care should therefore be taken in converting these values. Because of base-pair specificity effects, the fluorochrome employed for the DNA content measurement should always be identified.

13. Carry out ploidy analysis if desired.

    Ploidy analysis represents a special case in which the DNA contents of the unknown plant nuclei are compared to those from the same species having known ploidy.This comparison can be made either between two analyses performed under identical conditions, or, in some cases, through mixing the two (or more) samples for simultaneous measurement.

14. Carry out cell-cycle analysis, if desired, using cell-cycle analysis software.

    Cell-cycle analysis can be conveniently done on uniparametric flow histograms of plant nuclear DNA contents using commercially available software. This assumes an absence of endoreduplication. Alternatively, the proportions of cells within the various phases of the cell cycle can be determined by using a scissor to cut out from the appropriate plots the various areas corresponding to G_1, S, and G_2 cells, and weighing the paper. This approach can also be used for analysis of endoreduplicated cells, in which case the graphing of logarithmic uniparametric plots is required.

ANALYSIS OF SOMATIC DNA CONTENT IN PLANT TISSUES USING AN ARC LAMP–BASED FLOW CYTOMETER

The following alternate protocols are all for use with arc lamp–based flow cytometers and involve staining with 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI). Alternate Protocol 1 is for isolation of nuclei using lysis buffer LB01 (Dolezel et al., 1989), which works well with most plant species and tissues. For a very few species, resolution of DNA histograms is not satisfactory using LB01. In those cases, Alternate Protocol 2, which is a modification of a procedure originally developed by Otto (1990), can provide improved resolution (also see Dolezel and Göhde, 1995). The disadvantages of Alternate Protocol 2 are the it is quite laborious and that it is not universally applicable (i.e., some species give histograms with increased levels of background and high CVs). If none of these procedures works, the authors recommend the use of Partec buffer (de Laat et al., 1987). This buffer gives very good resolution with *Arabidopsis thaliana* tissues. The procedure for analysis using Partec buffer is identical to that for LB01 buffer (see Alternate Protocol 1).

The flow cytometer should be set up according to the manufacturer's instructions for analysis of PI- or DAPI-derived fluorescence. The steps described below for preparing the flow cytometer are for Partec flow cytometers.

Nuclear DNA Content Analysis Using LB01 Buffer or Partec Buffer and Arc Lamp–Based Flow Cytometer

ALTERNATE PROTOCOL 1

The following instructions are for preparation of cells with either LB01 (Dolezel et al., 1989) or Partec buffer (de Laat et al., 1987).

Additional Materials *(also see Basic Protocol)*

Lysis buffer LB01 *or* Partec buffer (see recipes) with fluorochrome, ice-cold
1 mg/ml propidium iodide (PI) stock solution (see recipe) *or* 0.1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI) stock solution (see recipe) and 1 mg/ml ribonuclease (RNase) stock solution (see recipe)

Particles for instrument alignment: e.g., microspheres or fixed chicken red blood cells (CRBCs; see Support Protocol 2) stained with the fluorochrome used (also see *UNIT 1.3*)

Glass petri dish
42-µm pore-size nylon mesh
Flow cytometer equipped with high-pressure mercury-arc lamp
Filters and dichroic mirror appropriate for fluorochrome used

Prepare suspensions of nuclei

1a. *For intact plant tissue, cell cultures, or callus: Weigh a small amount of plant material (typically 20 mg) and chop with a new razor blade or a sharp scalpel in 1 ml of ice-cold fluorochrome-containing LB01 lysis buffer or* Partec buffer in a glass petri dish (see Basic Protocol, step 1a, for chopping technique).

It is preferable to include the DNA fluorochrome (DAPI or PI) in the buffer. Alternatively, the stain may be added immediately after filtration (step 2). Note that commercially available Partec buffer contains 4 µg/ml DAPI.

The actual quantity of plant material to be used for nuclei isolation depends both on the type of tissue and on the species, and must be determined experimentally (larger quantities are usually needed for callus or cultured cells).

1b. *For protoplasts:* Resuspend protoplasts in ice-cold fluorochrome-containing LB01 lysis buffer *or* Partec buffer to a concentration of 1×10^5 to 1×10^6 protoplasts/ml.

For protoplasts the concentration of detergent (Triton X-100) in LB01 buffer should be increased to 0.5% (v/v); this improves the release of the nuclei from the protoplasts.

Nuclei cannot be released from "collapsed" protoplasts; hence protoplast viability is an important consideration. Typically the protoplasts should be 90% to 100% viable as determined using FDA (Harkins et al., 1990).

2. Filter the suspension through a 42-µm nylon mesh.

If the DAPI or PI was not initially added to the LB01 or Partec buffer, add 0.1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI) stock solution (see recipe) to a final concentration of 2 µg/ml or 1 mg/ml propidium iodide (PI) stock solution (see recipe) to a final concentration of 50 µg/ml along with 1 mg/ml RNase stock solution (see recipe) to a final concentration of 50 µg/ml.

3. Store on ice prior to analysis (a few minutes to 1 hr).

Set up and align the flow cytometer

4. Empty the waste container of the flow cytometer and fill the sheath-fluid container with distilled water.

Older Partec cytometers (e.g., PAS II) ran under vacuum and sheath fluid had to be deaerated. This is not necessary with the newer models.

5. Choose the optical filter set that corresponds to the fluorochrome used to stain nuclear DNA.

For DAPI-stained samples, use UG1 as the excitation filter, TK420 as the dichroic mirror, and GG435 as a barrier filter (a dry 20× objective is sufficient for most applications). For PI-stained samples, use BP520 as an excitation filter, TK575 as the dichroic mirror, and RG590 as a barrier filter; the use of a 40×/1.25 glycerol-immersion objective is recommended.

6. Set the amplification to linear mode.

7. Prepare particles suitable for instrument alignment (e.g., microspheres or fixed CRBCs stained with appropriate fluorochrome; see Support Protocol 2 and *UNIT 1.3*).

8. Using the particles prepared in step 7 and a low data rate (20 to 50 events/sec), adjust the gain of the instrument so that the peak corresponding to the G_1 nuclei appears approximately at one-fifth of the distance across the *x* axis (e.g., channel 50 on a 256 scale).

   It is important to run the analyses at a low data rate to achieve the highest resolution.

9. Check the resolution (the population coefficients of variation of the fluorescent peaks) and the linearity of the instrument.

   For CRBC nuclei, the coefficient of variation should be <2% (1% after DAPI staining). Because of the occurrence of clumped nuclei (e.g., doublets and triplets), fixed CRBC nuclei are very useful for checking instrument linearity.

10. If necessary, adjust the objective focus, the flow cell position, and/or the lamp focus and position to achieve the highest resolution.

11. Rinse the calibration particles from the sample line with water to avoid contamination of subsequent samples.

    The new generation of lamp-based Partec cytometers are very stable after initial alignment, and usually no further adjustment is needed.

Analyze the suspension of stained nuclei

12. Begin analyzing the nuclei (from step 3) at a rate of 20 to 50 nuclei/sec using linear amplification.

    Linear is preferred over logarithmic amplification for most applications. However, logarithmic amplification may be advantageous for the analysis of phenomena where a broad range DNA contents is expected (e.g., polysomaty or endoreduplication).

13. Adjust the gain of the instrument so that the peak corresponding to the G_1 nuclei appears approximately at one-fifth of the distance across the *x* axis (e.g., channel 50 on a 256 scale).

    Depending on the requirements of different types of experiment, the G_1 peak may be moved to different channel positions.

14. Acquire data for a total of 5,000 to 20,000 nuclei.

15. Analyze data (see Basic Protocol, steps 11 to 14).

Two-Step Nuclear DNA Content Analysis Using Arc Lamp-Based Flow Cytometer

ALTERNATE PROTOCOL 2

The following is a two-step procedure involving successive use of two different buffers (Otto, 1990; Dolezel and Göhde, 1995). Large numbers of samples can be prepared and simultaneously centrifuged (step 3). If necessary, the protocol can be interrupted after step 4 and the samples kept at room temperature for prolonged periods of time before continuing with addition of Otto II buffer and analysis.

Additional Materials *(also see Basic Protocol)*

Otto I buffer (see recipe) and Otto II buffer (with fluorochrome; see recipe)
1 mg/ml propidium iodide (PI) stock solution (see recipe) *or* 0.1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI) stock solution (see recipe) and 1 mg/ml ribonuclease (RNase) stock solution (see recipe)

Glass petri dish
42-µm pore-size nylon mesh
Tabletop centrifuge
Flow cytometer equipped with high-pressure mercury-arc lamp

Additional reagents and equipment for analysis with an arc lamp–based flow cytometer (see Alternate Protocol 1, steps 4 to 15)

1a. *For intact plant tissue, cell cultures, or callus:* Weigh a small amount of plant material (typically 20 mg) and chop with a new razor blade or a sharp scalpel in 1 ml of ice-cold Otto I buffer in a glass petri dish (see Basic Protocol, step 1a, for chopping technique).

1b. *For protoplasts:* Resuspend protoplasts in ice-cold Otto I buffer to a concentration of 1×10^5 to 1×10^6 protoplasts/ml.

2. Filter the suspension through a 42-µm nylon mesh.

3. Centrifuge 5 min at $150 \times g$, 4°C. Remove supernatant, leaving ~100 µl of liquid above the pellet, which contains the nuclei.

4. Resuspend the nuclei by gentle shaking, then add 100 µl of fresh Otto I buffer.

5. Incubate 10 to 60 min at room temperature with occasional shaking.

   The optimal incubation period depends on the plant species. Select the incubation period that gives the lowest background and CV.

6. Add 1 ml of Otto II buffer with fluorochrome. Hold sample at room temperature.

   It is preferable to include the fluorochrome (DAPI or PI) in the Otto II buffer at this step. Alternatively, these compounds can be added to the sample as the DAPI or PI/RNA stock solutions (see recipes) after the Otto II buffer has been added.

7. Analyze by flow cytometry within 5 to 15 min (see Alternate Protocol 1, steps 4 to 15).

ALTERNATE PROTOCOL 3

DNA CONTENT ANALYSIS OF FIXED PROTOPLASTS

In some cases, it is not possible to analyze plant material immediately after collection; hence a procedure is necessary that permits storage of material for analysis at later date. This is especially critical in experiments involving the measurement of cell-cycle kinetics, where samples have to be collected and analyzed at specific time intervals. This protocol describes preparation of fixed protoplasts.

Because of changes in chromatin structure, nuclei isolated from fixed tissues are not recommended for determination of DNA content in absolute units (genome size).

Additional Materials *(also see Basic Protocol)*

AES fixative (see recipe), ice-cold
70% ethanol
Homogenization buffer (see recipe)

Tabletop centrifuge
60-µm pore-size nylon mesh

1. Prepare protoplasts according to methods specific for the tissue and species of interest. Purify the protoplasts using gradient centrifugation. Centrifuge 5 min at $50 \times g$, 4°C, to obtain a pellet containing 1×10^5 to 1×10^6 protoplasts.

   Harkins et al. (1990) provides information about protoplast preparation and gradient purification.

2. Remove supernatant, then fix protoplasts by gently resuspending in 2 ml ice-cold AES fixative and incubating 5 min on ice. Centrifuge 5 min at $50 \times g$, 4°C, to recover protoplasts.

3. Repeat step 2, then wash twice, each time by removing the supernatant, adding 5 to 10 ml ice-cold 70% ethanol, then centrifuging 5 min at 50 × *g*, 4°C.

   Fixed protoplasts can be stored in 70% ethanol for up to a week at 4°C.

4. Remove supernatant and resuspend pellet in 2 ml homogenization buffer.
5. Filter the suspension through a 60-µm nylon mesh.
6. Stain and analyze the fixed protoplasts (see Basic Protocol or Alternate Protocol 1).

DNA CONTENT ANALYSIS OF FORMALDEHYDE-FIXED TISSUES AND CELLS

ALTERNATE PROTOCOL 4

This procedure for fixation and staining of plant tissues and cells is from Sgorbati et al. (1986).

Additional Materials *(also see Basic Protocol and Alternate Protocol 1)*

Formaldehyde fixative (see recipe), 4°C
Tris buffer (see recipe), 4°C
Lysis buffer LB01 (see recipe) without fluorochrome, 4°C
0.1 mg/ml DAPI stock solution (see recipe).

Tabletop centrifuge
Glass petri dish
42-µm pore size nylon mesh

1. Add 20 ml formaldehyde fixative to 10 to 100 mg plant tissue and incubate 10 min at 4°C.

   The optimal concentration of formaldehyde and the duration of fixation should be determined empirically for any given material (to achieve the highest possible resolution of peaks in the DNA content histograms).

2. Decant formaldehyde fixative and remove residual fixative with a Pasteur pipet. Wash out formaldehyde fixative by incubating in three changes of Tris buffer, each time for 10 min at 4°C.

   The fixed tissues can be stored at 4°C for up to several days. For prolonged storage, it is preferable that the material be stored as fixed nuclei rather than fixed tissue (see step 5).

3. Homogenize the tissues by crushing with a glass rod in 1 ml ice-cold Tris buffer or lysis buffer LB01 in a glass petri dish.

   Alternatively, isolate nuclei by chopping the tissues with a new razor blade and/or scalpel (see Basic Protocol). It is also possible to use a motorized homogenizer (e.g., Polytron PT 1200 from Brinkmann). In this case, transfer fixed tissues to a 12 × 75–mm polystyrene tube containing ice-cold LB01 buffer. This approach is especially convenient for isolation of nuclei from very small root tips and/or small amounts of cultured cells.

4. Filter the suspension through 42-µm nylon mesh to isolate nuclei.
5. Store the nuclei at 4°C prior to analysis.

   Fixed nuclei can be stored for more than a week.

6. Add DAPI stock solution to a final concentration of 2 µg/ml.

   Binding of propidium iodide to DNA in formaldehyde-fixed chromatin is impaired and the use of DAPI for DNA staining is recommended. Alternatively, the negative effect of the fixation may be reversed by heating (Overton and McCoy, 1994) and the nuclei stained with PI.

7. Analyze relative DNA content of isolated nuclei (see Basic Protocol, steps 4 to 14 or Alternate Protocol 1, steps 4 to 15).

ALTERNATE PROTOCOL 5

DNA CONTENT ANALYSIS OF FIXED, INTACT CELLS

This sample preparation technique may be used with plant tissues or isolated plant cells (Pfosser, 1989).

Additional Materials *(also see Basic Protocol or Alternate Protocol 1)*

Ethanol/acetic acid fixative (see recipe), ice-cold
70% ethanol, ice-cold
Citrate buffer (see recipe), 4°C
Enzyme solution (see recipe)
0.1 mg/ml DAPI stock solution (see recipe).
Tabletop centrifuge

1. Transfer the plant material (tissues or cells) into an excess of ice-cold ethanol-acetic acid fixative.
2. Centrifuge cells 5 min at 100 × *g*, 4°C. After centrifugation, replace the fixative and continue fixing for at least 8 hr at 4°C.
3. Wash cells twice, each time by centrifuging at 100 × *g*, 4°C, removing the supernatant, adding ice-cold 70% ethanol, then centrifuging again and removing the supernatant. Store fixed cells in 70% ethanol at −20°C.
4. Centrifuge cells 5 min at 100 × *g*, 4°C. Wash cells three times, each time by removing the supernatant, adding 4°C citrate buffer, then centrifuging 5 min at 100 × *g*, 4°C, and removing the supernatant.
5. Resuspend cell pellet in enzyme solution and incubate 2 hr at 37°C to release the nuclei.

   The concentration of enzymes and the duration of incubation should be determined empirically to achieve the highest resolution of DNA content histograms.
6. Centrifuge 5 min at 100 × *g*, 4° C, and remove enzyme solution.
7. Add DAPI stock solution to citrate buffer for a final concentration of 1 µg/ml. Using this solution, resuspend the cell pellet at a concentration of 1 × 10^6 nuclei/ml. Let sample stand 5 min prior to analysis.
8. Analyze relative fluorescence intensity via flow cytometry (see Basic Protocol, steps 4 to 14, or Alternate Protocol 1, step 4 to 15).

ALTERNATE PROTOCOL 6

BULK PLOIDY SCREENING

Some applications of flow cytometric ploidy determination involve large populations of plants. In order to certify the ploidy homogeneity of seed lots, random seed samples are taken and germinated, and the ploidy status of the plants determined. It is inefficient to measure each plant individually, and for the purposes of certification, a pooling strategy can be employed. Various numbers of the germinated plants are homogenized together and the mixed populations of nuclei are analyzed. Since nuclei of different ploidies (e.g., diploid and triploid) are well separated on one-dimensional DNA histograms, as long as sufficient nuclei are analyzed one can confidently determine the relative proportions of the two ploidy classes in the homogenates.

1. Germinate seeds under appropriate conditions.

   If required, the seeds can be surface-sterilized by immersion for 15 min in diluted (30% v/v) commercial bleach solution. The residual bleach is removed by washing the seeds several times in sterile water.

2. Select seedlings at the appropriate growth stage and excise tissue samples. Pool equal fresh weights of the sampled tissues.

   The numbers of plants that can be pooled should be determined empirically, using individuals of known ploidies. Thus, one triploid plant tissue sample should be mixed with various numbers of diploid plant samples, or vice versa, prior to homogenization.

3. Subject the plant samples to homogenization and flow analysis (see Basic Protocol or Alternate Protocol 1). Examine the resultant DNA histograms to determine the numbers of nuclei (hence time of analysis) required for accurate detection of the minor ploidy class.

   From these measurements, the most efficient strategy for ploidy determination can be devised.

PREPARATION OF UNFIXED CHICKEN RED BLOOD CELLS FOR USE AS INTERNAL STANDARDS IN GENOME-SIZE MEASUREMENTS

SUPPORT PROTOCOL 1

Materials

Live chicken
Acid citrate dextrose (ACD) buffer (see recipe)
Dimethylsulfoxide (DMSO)

21-G needle
Heparinized blood collection tube
Tabletop centrifuge
Polypropylene cryotubes (e.g., Nunc)
Liquid nitrogen

1. Using a 21-G needle, collect 10 ml of venous blood in a heparinized tube from a blood vessel under the wing of a healthy chicken. Mix the tube by gently inverting and place on ice.
2. Centrifuge 10 min at 500 × g, 4°C.
3. Aspirate plasma and gently resuspend pellet in 10 ml ACD buffer.
4. Centrifuge 10 min at 500 × g, 4°C. Aspirate supernatant and resuspend pellet in 10 ml ACD buffer. Repeat centrifugation and resuspend pellet again in ACD buffer.
5. Add 0.8 ml DMSO to resuspended cells and mix gently.
6. Pipet 100-µl aliquots into cryotubes and freeze in liquid nitrogen. Store at –80°C up to one year.
7. When ready to use, thaw aliquot on ice and add directly to sample as a reference standard.

   CRBCs may be diluted in sample buffer if necessary.

SUPPORT PROTOCOL 2

PREPARATION OF FIXED CHICKEN RED BLOOD CELL NUCLEI FOR INSTRUMENT ALIGNMENT

This procedure was developed by J. Dolezel (unpub. observ.). For additional discussion of the use of particles for instrument alignment, see *UNIT 1.3*.

Materials

Fresh chicken blood (collected in heparinized tube to prevent coagulation)
CRBC buffers I, II, and III (see recipes)
Ethanol/acetic acid fixative (see recipe), ice-cold
70% ethanol, ice-cold

Tabletop centrifuge
15-ml polypropylene centrifuge tubes
30-G needle and syringe
42-µm pore-size nylon mesh

1. Mix 1 ml fresh chicken blood with 3 ml CRBC buffer I in a 15-ml tube. Centrifuge at 5 min at 50 × *g*, 4°C.
2. Discard the supernatant, add 3 ml CRBC buffer I, and gently mix. Centrifuge 5 min at 50 × *g*, 4°C.
3. Discard the supernatant, resuspend the pellet in 2 ml CRBC buffer II, and vortex briefly.
4. Immediately add 2 ml CRBC buffer III and vortex briefly.
5. Centrifuge 5 min at 250 × *g*, 4°C. Discard the supernatant, add 2 ml CRBC buffer III, and mix gently.
6. Centrifuge 5 min at 120 × *g*, 4°C. Discard the supernatant. Transfer the pellet to a clean 15-ml tube, add 2 ml CRBC buffer III, and mix gently.
7. Centrifuge 5 min at 90 × *g*, 4°C. Discard the supernatant.
8. Resuspend the pellet in the residual supernatant, add 2 ml ice-cold freshly prepared ethanol/acetic acid fixative, and vortex briefly.
9. Leave overnight at 4°C. Do not shake.
10. Gently remove the fixative and resuspend the pellet of nuclei in residual fixative.
11. Add 6 ml ice-cold 70% ethanol, vortex briefly, and pass three times through a 30-G needle using a syringe.
12. Filter the nuclear suspension through a 42-µm nylon mesh to remove large clumps.
13. Store in 2-ml aliquots in 70% ethanol at −20°C.

    If the concentration of nuclei is too high, dilute with ice-cold 70% ethanol before storage.

    Isolated nuclei can be stored for several years without any sign of deterioration.

    IMPORTANT NOTE: *Fixed nuclei are not suitable as a standard for estimation of DNA content in absolute units (genome size).*

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acid citrate dextrose (ACD) buffer

136 mM glucose
75 mM trisodium citrate
4.2 mM citric acid monohydrate
Filter through 0.22-µm filter
Prepare fresh

AES (acetic acid/ethanol/sorbitol) fixative

Prepare a solution of 18 parts ethanol:3 parts acetic acid:1 part water. Dissolve sorbitol in this solution to a 1% (w/v) final concentration. Prepare fresh.

Recipe from Galbraith and Shields (1982).

Citrate buffer

10 mM disodium EDTA
10 mM sodium citrate
Adjust pH to 4.8
Prepare fresh

CRBC buffer I

140 mM NaCl
10 mM sodium citrate
1 mM Tris·Cl, pH 7.1 (*APPENDIX 2A*)
Prepare fresh

CRBC buffer II

140 mM NaCl
5% (v/v) Triton X-100
Prepare fresh

CRBC buffer III

320 mM sucrose
15 mM $MgSO_4 \cdot 7\ H_2O$
15 mM 2-mercaptoethanol
1 mM Tris·Cl, pH 7.1 (*APPENDIX 2A*)
Prepare fresh

DAPI stock solution

Prepare a 0.1 mg/ml solution of 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Filter through a 0.22-µm filter to remove small particles. Store up to 6 months at −20°C in 1-ml aliquots.

Enzyme solution

Citrate buffer (see recipe) containing:
0.5% (v/v) cellulase Onozuka R10 (Serva)
5% (v/v) pectinase from *Aspergillus niger* (Sigma)
1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes)
Prepare fresh

Ethanol/acetic acid fixative

Mix 3 parts 96% ethanol with 1 part glacial acetic acid. Prepare fresh.

Formaldehyde fixative

Add formaldehyde to freshly prepared Tris buffer (see recipe) to a final concentration of 4% (v/v). Prepare fresh.

Homogenization buffer

45 mM $MgCl_2$
20 mM 3-(*N*-morpholino) propane sulfonate (MOPS)
30 mM sodium citrate
0.1% (v/v) Triton X-100
Adjust pH to 7.0 with NaOH
Filter through 0.22-µm filter
Store up to 6 months frozen at −20°C

This recipe is from Galbraith et al. (1983). Certain additives may be helpful in suppressing interference from the presence of secondary products in some plant species; see Basic Protocol, step 10 annotation.

Lysis buffer LB01

15 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
2 mM disodium EDTA
0.5 mM spermine tetrahydrochloride
80 mM KCl
20 mM NaCl
0.1% (v/v) Triton X-100 (0.5% for protoplasts)
Adjust to pH 7.5 with 1 M NaOH
Filter through 0.22-µm filter
Add 2-mercaptoethanol to 15 mM
Store up to 6 months at in 10-ml aliquots at −20°C

Optional: Prior to use, add 0.1 mg/ml DAPI stock solution (see recipe) to a final concentration of 2 µg/ml *or* 1 mg/ml PI stock solution (see recipe) to a final concentration of 50 µg/ml along with 1 mg/ml RNase stock solution (see recipe) to a final concentration of 50 µg/ml.

Recipe is from Dolezel et al. (1989). Alternatively, the DAPI or PI/RNase may be added to the cell suspension after it has been filtered.

Mithramycin (MI) stock solution

Prepare 0.1 mg/ml mithramycin (Sigma) in homogenization buffer (see recipe). Filter through a 0.22-µm filter. Store up to 6 months in 0.5-ml aliquots at −20°C.

Different samples of commercial mithramycin vary in purity. The optical density of the solution at 420 nm should be checked and adjusted to 0.6 with additional mithramyicin if necessary.

Otto I buffer

0.1 M citric acid
0.5% (v/v) Tween 20
Filter through a 0.22-µm filter
Prepare fresh

Otto II buffer

Prepare 0.4 M $Na_2HPO_4 \cdot 12H_2O$. Filter through a 0.22-µm filter; store up to 6 months at room temperature. Prior to use (if stain is to be incorporated directly in the buffer), add 0.1 mg/ml DAPI stock solution (see recipe) to a final concentration of 4 µg/ml *or* 1 mg/ ml PI stock solution to a final concentration of 50 µg/ml along with 1 mg/ml RNase stock solution (see recipe) to a final concentration of 50 µg/ml.

RNase is needed to prevent PI from binding to RNA. Since DAPI specificially stains DNA RNase is not needed with that dye (see Background Information).

Partec buffer

0.2 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
4 mM $MgCl_2$
0.5% (v/v) Triton X-100
Prepare fresh

Optional: Prior to use, add 0.1 mg/ml DAPI stock solution (see recipe) to a final concentration of 2 µg/ml *or* 1 mg/ml propidium iodide PI stock solution (see recipe) to a final concentration of 50 µg/ml along with 1 mg/ml RNase stock solution (see recipe) to a final concentration of 50 µg/ml.

RNase is needed to prevent PI from binding to RNA. Since DAPI specificially stains DNA RNase is not needed with that dye (see Background Information).

Recipe is from de Laat et al. (1987). Note that the commercially available Partec buffer contains 4 µg/ml DAPI.

Propidium iodide (PI) stock solution, 1 mg/ml

Prepare 1 mg/ml propidium iodide (PI; Molecular Probes). Filter through a 0.22-µm filter. Store up to 6 months in 0.5-ml aliquots at −20°C.

RNase stock solution, 1 mg/ml

Prepare a 1 mg/ml solution of RNase (type IIA, Sigma). Heat 15 min at 90°C to inactivate DNases. Filter through a 0.22-µm filter. Store up to 6 months in 0.5-ml aliquots at −20°C.

Tris buffer

100 mM NaCl
10 mM disodium EDTA
0.1% (v/v) Triton X-100
10 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
Prepare fresh

COMMENTARY

Background Information

Eukaryotic organisms are characterized by the envelopment of the cellular genetic material within organelles. In interphase cells, the majority of this material is found in the nucleus in the form of linear double-stranded DNA molecules, organized within hereditary units termed chromosomes. With the development of methods for the measurement of nuclear DNA content, it has long been recognized that these values are tightly controlled and are characteristic of the species in which they are measured. In most cases, different somatic cell types have a constant value for nuclear DNA content, which is twice that of the gametes. From this has come the definition of the somatic DNA content (2C). The between-species variation in C value is particularly striking in the higher plants—from a low of ~0.15 pg in *Arabidopsis thaliana* to as much as 127.4 pg in *Fritillaria assyriaca*—and may have evolutionary significance (Bharathan et al., 1994).

If it is assumed that the gametes are haploid (for higher plants this may not always be the case—e.g., as a result of polyploidization during speciation), then diploid somatic cells have a 2C DNA content. Measurement of DNA content values and comparison to known standards can therefore provide an assessment of ploidy. Ploidy analysis turns out to have considerable importance in agriculture. Particularly in Europe, commercial seed production requires certification of the presence of a single ploidy class in the seed. For some species (e.g., soybeans and hybrid maize) this requirement is superfluous, either because of the breeding system or the reproductive biology of the plant. For other species, the breeding strategy leading to seed production may require that seed producers plant only seeds of a specific ploidy class (triploid seedless watermelon and sugar beet are two such examples). Certification of ploidy homogeneity in these cases is critical. A new application of ploidy analysis has recently emerged in the field of plant genetic engineer-

ing. The regeneration of transformed crop plants, or of chemically induced doubled haploids, frequently provides populations many of which are non-euploid off-types. Maintenance of these undesirable plants in greenhouses constitutes a considerable economic sink. The availability of flow cytometric techniques to efficiently and accurately rogue out non-diploids at an early stage is of considerable benefit. Finally, the accuracy with which nuclear DNA contents can be measured has led to use of flow cytometry to identify aneuploidy (Pfosser et al., 1995). This should be important in a variety of molecular breeding strategies, as well as for the isolation of individual chromosomes through flow sorting.

Flow cytometric analysis can also be extremely useful for analysis of the cell-division cycle in plants, which is thought to operate in a manner similar to that seen in other eukaryotes. Somatic cells having a 2C DNA content are in either G_0 phase (noncycling) or G_1 phase (cycling). Cycling cells subsequently enter S phase, wherein the nuclear DNA content is doubled, and then G_2 phase. This is followed by mitosis and cell division. Analysis of the nuclear DNA content via flow cytometry provides an accurate estimate of the proportions of the cells within the various phases of the cell-division cycle, although it can be difficult to distinguish cells in G_0 from those in G_1. In general, the flow histograms of plant tissues show a major peak of G_0/G_1 cells, a well defined G_2 peak, and few cells in S phase, even for rapidly growing cell cultures.

An additional complication in the analysis of flow histograms from plant tissues is the occurrence of somatic endoreduplication (DeRocher et al., 1990; Galbraith et al., 1991). This involves multiple occurrences of S phase without intervening mitoses, and results in the presence of discrete populations of cells having DNA contents in the series 2C, 4C, 8C, 16C, and so on. Somatic endoreduplication is found in specialized situations (e.g., the maize endosperm), but can also be found in "normal" tissues, such as leaf epidermis and mesophyll in *Arabidopsis thaliana*. Analysis of endoreduplication requires the use of logarithmic abscissas, and current software programs do not allow calculation of the proportions of cells within the various endoreduplicative cycles. In any case, distinguishing 4C cells in G_2 of the diploid cell cycle from G_1 cells in the first endoreduplicative cycle is problematic.

The knowledge of DNA content in absolute units (genome size) is important in many areas of research ranging from evolutionary studies to genome mapping. Flow cytometry is a very convenient approach to determining genome size in plants. However, some precautions should be taken in this application. It has already been mentioned that DAPI and mithramycin exhibit a pronounced base-pair specificity in DNA binding. In calculation of absolute DNA contents in comparison to internal standards such as CRBCs, it is clear that use of these fluorochromes will introduce systematic errors that in some cases can be quite large. Godelle et al. (1993) have described methods whereby this feature can be employed for the analysis of genome base-pair ratios.

The various fluorochromes differ in the manner in which they bind to nucleic acids. Ethidium bromide and PI bind through intercalation. In order to measure DNA contents, treatment with RNase is required to eliminate fluorescence contributed by binding to RNA. Hoechst 33258, DAPI, and MI specifically bind DNA and not RNA; hence RNase treatment is not required. There is a disadvantage, however, in using these dyes. Unlike ethidium bromide and propidium iodide, they exhibit base-pair specificity in binding to DNA, and this must be remembered if they are to be used for estimation of genome size. On the other hand, the results obtained with different fluorochromes can be used to estimate AT/GC ratios. DAPI typically provides DNA histograms having G_1 peaks with the lowest coefficients of variation, and is a preferred fluorochrome for ploidy analysis, including the detection of aneuploidy.

It should also be noted that PI, being an intercalating dye, is sensitive to the degree of chromatin condensation. For some species, this property has been employed to explore the proportions of euchromatin and heterochromatin present in the genome (Rayburn et al., 1992). In all cases, it should be remembered that alterations in chromatin condensation as a function of growth state or tissue type might well affect total fluorescence emission (O'Brien et al., 1996). Thus it is recommended that comparative analysis between different samples employ tissues of similar metabolic and developmental state.

Critical Parameters and Troubleshooting

Sample Preparation

If the acceptance criterion is defined as a CV of <5%, many plant species provide acceptable DNA histograms. If unacceptably high CVs are

encountered, supplementing the homogenization medium with various components can be helpful. In particular, the authors have found that inclusion of antioxidants, bovine serum albumin (BSA), and polyvinylpyrrolidone (PVP) can be critical for obtaining adequate flow histograms from some species (Bharathan et al., 1994; Dolezel et al., 1994). Empirical analysis of the effects of varying the level of Triton X-100 in the homogenization medium over the range of 0.1% to 1% or even higher has also been recommended (S.C. Brown, pers. comm.).

It is critical that sample-staining conditions be uniform and that the fluorochrome saturate all available binding sites. Investigators should perform saturation binding curve measurements using the system of interest (Galbraith et al., 1983), and then adhere to these conditions in subsequent experiments. PI is known to bind to plastic surfaces, including the sample-introduction tubing. It is recommended that the flow cytometer be preequilibrated by passing an appropriate concentration of PI through the system before analyzing the samples of interest. Conversely, prior to further experiments, traces of PI can be removed from the flow cytometer by passing a 10% solution of commercial bleach through the system, followed by deionized water.

Standards

Use of standard fluorescent microspheres for setting up and aligning flow cytometers is critical and should be routinely employed. Furthermore, the authors consider essential the use of internal standards for applications involving comparative analysis of plant nuclear DNA contents. For large-scale screening of ploidy levels, internal standards may be less important. Sequential analysis of unknowns followed by plants of known ploidy status is usually sufficient to affirm cytometer stability. For measurements requiring greater precision—e.g., the detection of aneuploidy, an internal standard is essential.

The use of internal standards is also important in analysis of genome size. Various standards can be employed, including CRBCs, trout red blood cells, or plant homogenates having nuclei of known DNA content (Dolezel et al., 1992; Bharathan et al., 1994). For greatest consistency, it is recommended that the DNA contents of these standards be determined using some method that is independent of fluorescence (e.g., the diphenylamine procedure; Burton, 1968; Galbraith et al., 1983). Intraspecies variation in DNA content has been noted for some plant species (Rayburn et al., 1992) but not others (Baranyi and Greilhuber, 1996; Greilhuber and Obermayer, 1997). Flow cytometric analysis is usually characterized by a high degree of resolution, with CVs ranging from 1% to 3%. It should be noted that this precision is related to the accuracy of individual measurements and does not directly report the reproducibility of DNA-content estimation. It is thus important to perform sufficient independent experiments both to provide statistical significance and to uncover any unexpected variation.

Flow cytometry

In all cases, it should be remembered that the flow cytometric measurements aim to identify a minor subpopulation of fluorescent nuclei within a cellular homogenate that contains a large population of different particles, all of which scatter light and some of which are autofluorescent (e.g., chloroplasts). For laser-based instruments, it has already been noted that triggering based on fluorescence and not light scatter is required (see Basic Protocol). In some cases (e.g., tobacco), the peak of fluorescent nuclei is well separated from that of other fluorescent particles. This corresponds to the situation where the nuclear DNA content is quite large. In other species of interest, the nuclear DNA content can be much smaller (e.g., *Arabidopsis thaliana*). In this latter case, the contribution from chlorophyll autofluorescence can overlap PI-induced nuclear fluorescence on one-dimensional histograms. The use of biparametric (orange versus red) displays can be helpful, since the endoreduplicated series of nuclei can be readily identified and gates placed around these nuclei (Galbraith and Lambert, 1995).

It is recommended that flow histograms of nuclear fluorescence emission versus time be routinely collected. The major G_1 peak of fluorescence should be stable in intensity as a function of time. Aberrant events, discontinuities, and so on can provide an early warning of problems in staining stoichiometries or kinetics, or in flow cytometer function (e.g., a partially blocked flow cell tip) that might otherwise lead to unacceptably high CVs.

As a general rule, sufficient events should always be collected to provide unambiguous and statistically significant data. Typically, 5,000 to 20,000 nuclei should be analyzed for each sample.

Anticipated Results

A typical uniparametric display of the population fluorescence emission of PI-stained nuclei from a tobacco leaf homogenate is given in Figure 7.6.1A (Bharathan et al., 1994). The major peak is from G_1 nuclei having a 2C DNA content. A minor peak is observed at twice the fluorescence-intensity value (in this case, at about channel 800), corresponding to the G_2 nuclei. S-phase nuclei are found between these two peaks. Integration of the number of nuclei beneath each area of the histogram provides a description of the cell cycle status of the tissue.

For determination of the G_1 DNA content value, chicken red blood cells are included as an internal standard (Fig. 7.6.1B). In this case, an additional peak is observed (at about channel 100). Since the abscissa is linear, simple division of the peak positions allows calculation of the DNA content of the “unknown” by reference to the known DNA content of CRBCs. Use of *N. tabacum* as an internal standard is shown in Figure 7.6.2A. Here, the 2C peak for the unknown (*Alstroemeria caryophyllacea*) has a DNA content larger than that of *N. tabacum* and considerably larger than that of CRBCs

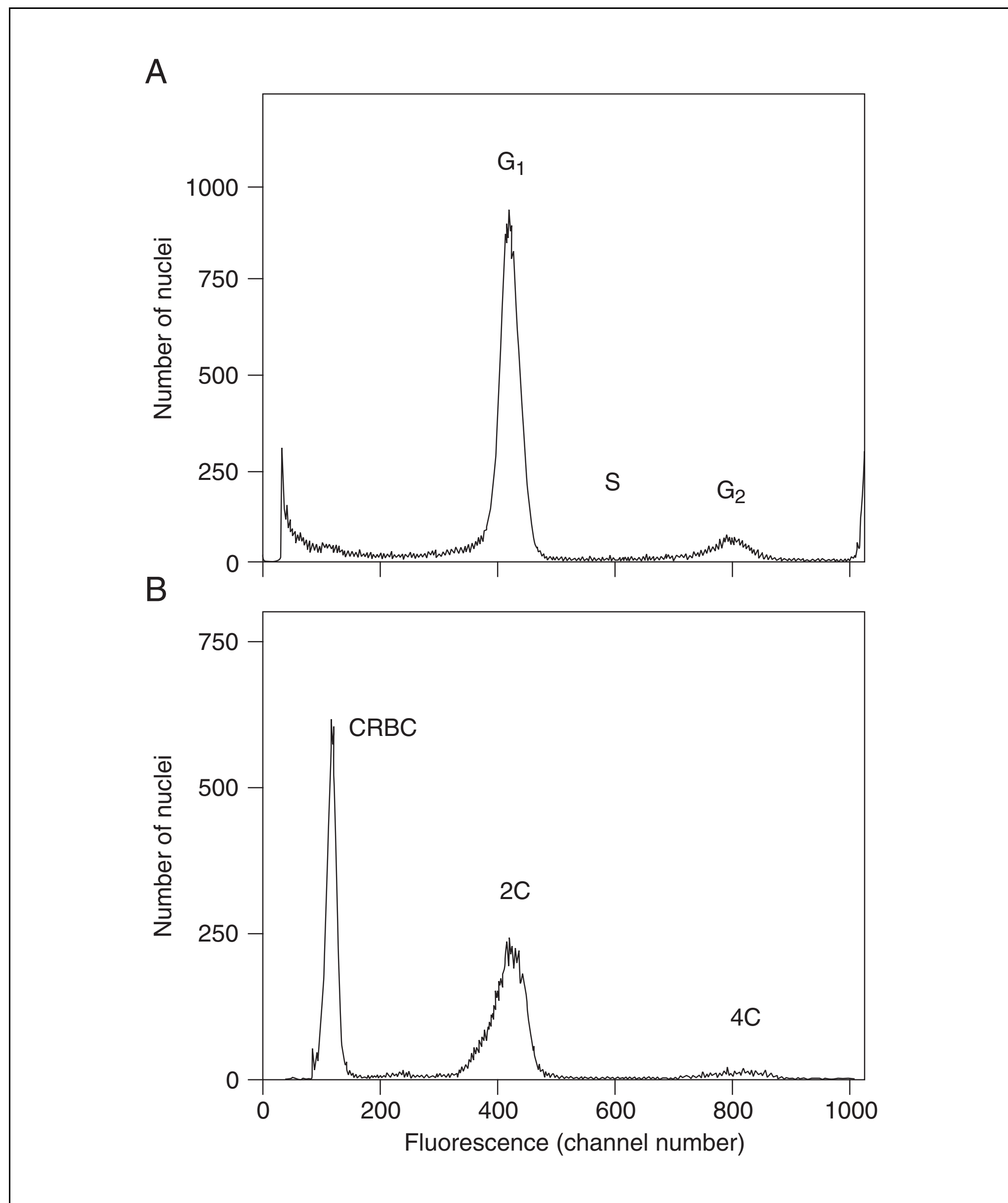

Figure 7.6.1 **(A)** Flow cytometric analysis of tobacco leaf homogenates stained with propidium iodide. **(B)** Same analysis with inclusion of chicken red blood cells (CRBC) as an internal standard for genome size measurement. In leaf tissue the G_1 nuclei, which have a 2C DNA content, greatly outnumber those in S phase and in G_2, which have a 4C DNA content.

(Bharathan et al., 1994). For accurate estimation of larger genome sizes, use of standards that are close to that of the unknown is advantageous. For situations in which large dynamic ranges are unavoidable, logarithmic amplification and display are helpful (Fig. 7.6.2B).

DNA histograms from samples having large genome sizes generally have little background, and the various peaks are discrete and well separated because the intensity of fluorescence, even employing PI, greatly exceeds that of any other type of particle in the homogenate. For species having much smaller genomes—e.g., *Arabidopsis thaliana*—the contribution of fluorescent particles can cause problems. Thus, the endogenous orange-red autofluorescence of pigments within *Arabidopsis* chloroplasts contributes a significant amount of background to the analysis of PI-stained nuclei. This can be ameliorated though use of gating on biparamet-

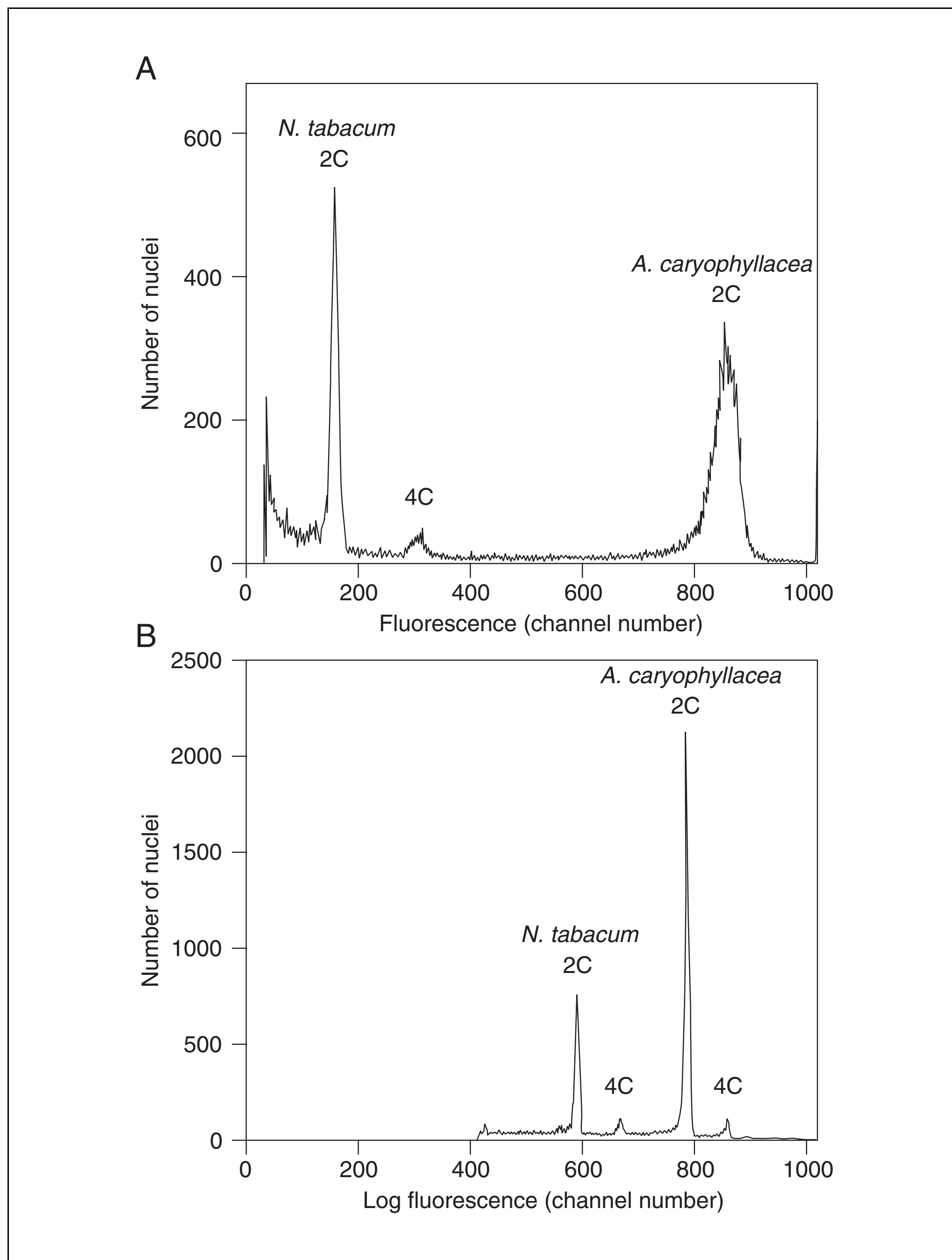

Figure 7.6.2 Analysis of the genome size of *Alstroemeria caryophyllacea* using *N. tabacum* as the internal standard and PI as the fluorochrome. **(A)** Employing linear amplification, only the G_1 (2C) peak for *A. caryophyllacea* is on scale. **(B)** Employing logarithmic amplification, G_1 and G_2 nuclei for both species are observed (from Bharathan et al., 1994).

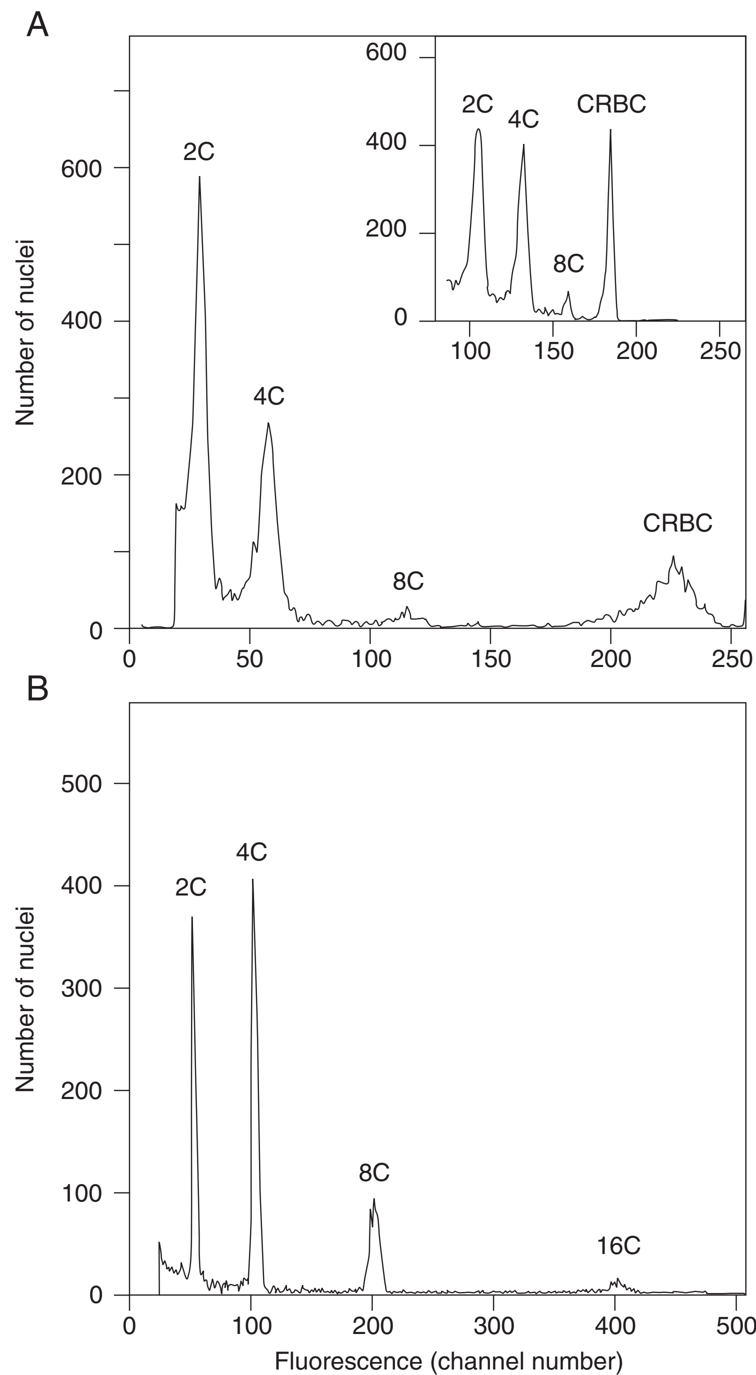

Figure 7.6.3 Flow cytometric analysis of nuclear DNA contents in young *Arabidopsis thaliana* leaf tissues. **(A)** Use of mithramycin as the fluorochrome and CRBCs as the internal standard to provide a measure of the genome size (insert represents the same sample under logarithmic amplification); from Galbraith et al., 1991. **(B)** Use of fluorochrome DAPI, Partec buffer, and the Partec PAS II flow cytometer. This gives particularly well separated and discrete peaks of fluorescence; the extensive somatic endoreduplication is readily observed and accurately measured.

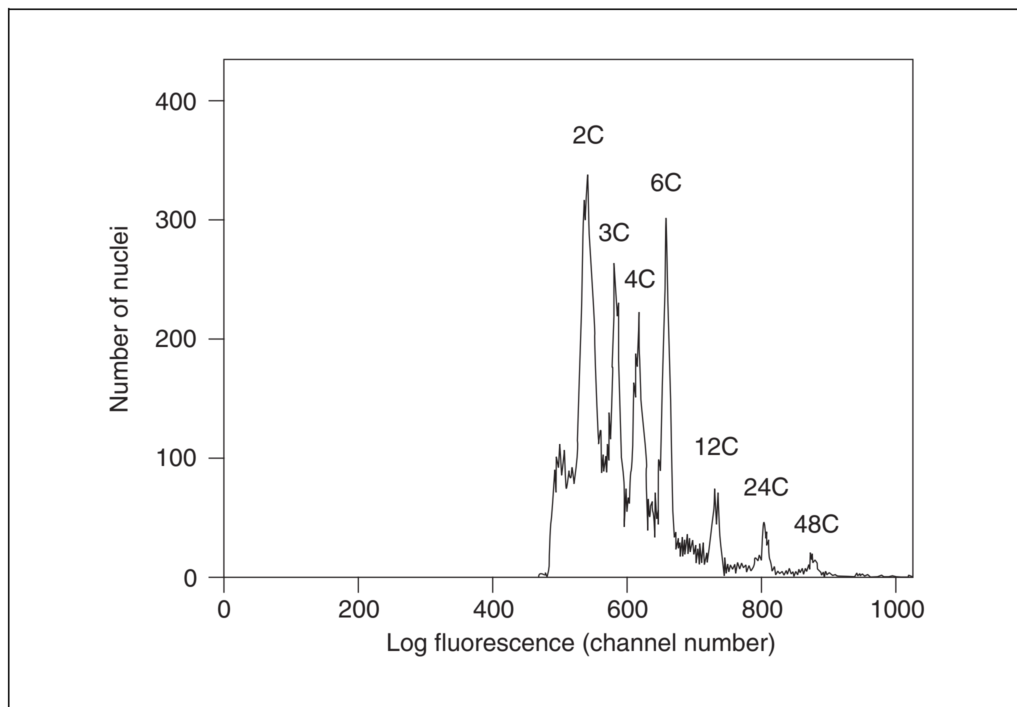

Figure 7.6.4 Flow cytometric analysis of nuclear DNA content distribution in maize endosperm (28 days after pollination) using PI as the fluorochrome (from Galbraith and Lambert, 1995).

ric displays, as previously described (see Critical Parameters). Alternatively, the emission spectrum of MI or DAPI is sufficiently removed from that of chlorophyll to largely eliminate this background (Fig. 7.6.3). Estimation of the genome size of *Arabidopsis* requires correction for the differences in AT/GC composition between *Arabidopsis* and CRBC nuclei (Galbraith et al., 1991).

An interesting observation within *Arabidopsis* is the occurrence of systemic endoreduplication (Galbraith et al., 1991), which is also observed in succulents having small genomes (DeRocher et al., 1990). Endoreduplication is a conspicuous feature of specialized storage tissues, notably the developing maize endosperm (Fig. 7.6.4), and results in nuclei with extraordinarily high C values (Kowles et al., 1990).

Time Considerations

Routine DNA analysis on intact plant tissues can be done by one person at a rate of 5 to 6 samples per hr. If the procedures are pipelined appropriately and an autosampling flow cytometer is employed, this can be increased to a maximum of ~20 samples/hr, since each sample requires ~2 min of flow cytometric data acquisition.

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Outlines the basic procedures for rapid analysis of plant nuclei using flow cytometry which, with various modifications, are now employed worldwide for a variety of different applications.

Internet Resources

Bennett, M.D., Cox, A.V., and Leitch, I.J. 1997. Angiosperm DNA C-values database. *http://www.rbgkew.org.uk/cval/database1.html*.

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