Chapter 12
Flow Cytometry: Cell Cycle

Teodoro Coba de la Peña and Adela M. Sánchez-Moreiras

1 The Plant Cell Cycle

Quantitative analyses of cell cycle can give essential information about the response of plants to short- or long-term abiotic or biotic stress, as most species alter leaf expansion or root growth as one of the first responses to cope with adverse environmental conditions (Boyer 1982). Tardieu and Granier (2000) observed a reduction of leaf area under water and light deficits due to partial blockage of nuclei in G1, which increased cell cycle duration and decreased final cell number. This effect can be detected shortly after the application of the stress and, sometimes, does not alter the photosynthetic rate, as is independent of carbon metabolism. Something similar happens with root development, where increasingly more works are focusing the interest on the study of auxin-regulated gene expression, the role of protein kinases as key regulators in plant growth and development, and the cell cycle rate and dynamic measurements in stressed tissues (Sánchez-Moreiras et al. 2006).

Flow cytometry makes possible a fine approach to the study of these events, including basic mechanisms of the cell cycle (rates of proliferating and quiescent cells, characterization of cell subsets and states upon cell cycle length and progression), and also study of effects of different putative modulators and inhibitors (hormones, growth factors, toxins, maybe allelochemicals, etc.) and environmental conditions (including stress) on the cell cycle.

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T. Coba de la Peña
Centro de Estudios Avanzados en Zonas Áridas (CEAZA), La Serena, Chile

A. M. Sánchez-Moreiras (✉)
Department of Plant Biology and Soil Science, University of Vigo, Vigo, Spain
e-mail: adela@uvigo.es

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Cell cycle rate and dynamics can be affected due to very diverse environmental conditions. As extensively reviewed by Granier and collaborators (2007), several factors can spatial and temporally affect cell division rate in the different organs of the plant in very different ways. In this respect, water deficit or reduced incident light cause a fall in mitotic activity decreasing the cell division rate in roots and shoots (Chiatante et al. 1997; Schuppler et al. 1998; Cookson and Granier 2006), while high temperature can reduce or increase the cell division rate depending on the range of temperatures and the equilibrium with the duration of the cell cycle (Granier et al. 2007).

Besides abiotic conditions, also contaminants (Wonisch et al. 1999); different pesticides, such as acetochlor or diquat (Chauhan et al. 1999; Freeman and Rayburn 2006); and other chemical compounds, such as secondary metabolites (Sánchez-Moreiras et al. 2008), were found to have a great impact on cell cycle, reducing the number of cells in division. Wisniewska and Chelkowski (1994) and Packa (1997, 1998) studied the potential genotoxicity of *Fusarium* mycotoxins on wheat cells, finding decreased mitotic index after the treatment, with excessive condensation of prophasic and metaphasic chromosomes, accumulation of metaphases and a significantly increase of the percentage of cells with chromosomal aberrations. As well, the secondary metabolite artemisinin, a highly phytotoxic compound produced by *Artemisia annua*, was also found to show abnormal metaphase and anaphase configurations (Dayan et al. 1999), and aryltetralin plant lignans induced inhibition at all phases of mitosis with abnormal star anaphase chromosomal configurations. The exact mechanisms of action of these compounds are still unknown, but a primary effect seems to be the alteration of the formation of the spindle microtubular organization centers, resulting in the formation of multiple spindle poles and an asymmetrical convergence of the chromosomes (Oliva et al. 2002). More recently, flow cytometric analyses and mitotic index showed a retard of cell cycle in lettuce meristems treated with Benzoxazolin-2(3H)-one (BOA), with selective activity at G2/M checkpoint (Sánchez-Moreiras et al. 2008). Blocking and delay of mitosis was also found on Arabidopsis root meristems after some minutes of citral treatment (Graña et al. 2013).

Although the plant cell cycle can be regulated at multiple points, biotic and abiotic stress seems to predominantly operate at the G1 to S and G2 to M transitions (Granier et al. 2007). Cells (and, in particular, the nuclei) can be at different possible states or phases (Marie and Brown 1993; Francis 2009; Scofield et al. 2014): G0, G1, S, G2 and M. The whole of G1, S and G2 phases is termed ‘interphase’.

- Cells in G0 phase (or Gap 0): cells in quiescent state after mitosis, i.e. differentiated or undifferentiated cells that do not divide and are not involved in active (proliferating) cell cycle events. This quiescent state can be reversible, and then cells enter in G1 phase.
- Cells in G1 phase: involved in cell growth and active cell cycle. They are characterized by a 2C nuclear DNA content (that is, with double DNA amount than that of gametes). This interval precedes nuclear DNA synthesis.

- Cells in S (Synthesis) phase: DNA synthesis takes place, and cell can duplicate progressively their nuclear DNA content.
- Cells in G2 phase, which is an interval between the end of DNA synthesis and the beginning of mitosis. They are characterized by a 4C nuclear DNA content.
- Cells in Mitosis (M): chromatin condenses, becoming chromosomes. Nuclear envelope disappears. Later on, chromosome segregation occurs, appearing new nuclear membranes, originating two daughter nuclei. This event is usually followed by cell division (cytokinesis). Thereby, this presently 4C cell divides in two 2C daughter cells.

Daughter cells can enter in G0 phase for a time, or enter directly in the G1 phase of a new cell cycle. In every described phase, cell cycle progression can stop and cell entries in a new quiescent phase. By this way, cells in quiescent G1, S and G2 phases (called $G1_Q$, S_Q and $G2_Q$, respectively) appear. Transition phases between quiescent and proliferating cells have also been described, and they are called $G1_T$, S_T and $G2_T$. G0 and $G2_Q$ can be followed by irreversible differentiation of the tissue cells, that do not divide anymore, although regression to undifferentiated and newly proliferating cells has sometimes been observed in mesophyll cells (Marie and Brown 1993).

If anomalous mitosis occurs (endomitosis, characterized by no formation of mitotic spindle and no attainment of chromosome segregation), a single nucleus with double number of chromosomes (corresponding to a 4C DNA content) becomes permanent. This event can take place several times, originating cells with a DNA content of 8C, 16C, 32C, 64C, etc., that is, with different ploidy levels. Endopolyploidy is originated by this way. In fact, this phenomenon is common in plants (Barow and Jovtchev 2007), and different tissues of a given plant organism can show different ploidy levels (polysomaty).

Length of each phase varies upon species, tissues and cell physiology. In a sample of proliferating cells (as is the case of plant meristems or some plant cell suspensions), most of them are in G1 phase, because this is the longest phase. The higher part of plant tissues is composed by fully differentiated (quiescent) cells, which do not divide anymore.

2 Flow Cytometry for Cell Cycle Analyses in Plants

In this section, we will describe the simplest modality of cell cycle analysis performed by flow cytometry.

Moreover, using simultaneously other fluorescent dyes and fluorescent-labeled monoclonal antibodies, RNA and protein content and synthesis, identification of antigens and molecular markers that are specific and/or critical of a cell cycle subphase can also be analyzed at the same time that the cell cycle.

Samples to be analyzed by flow cytometry are prepared from plant suspensions or meristems. Briefly, using a Petri dish, intact plant tissue is chopped with a razor

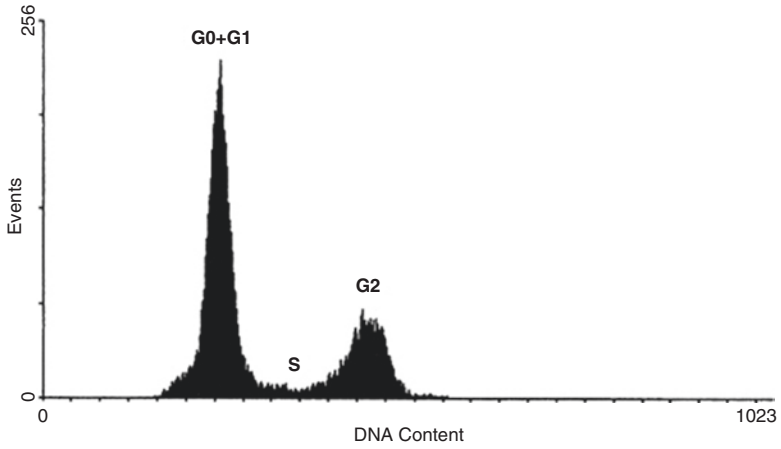

Fig. 12.1 Monoparametric linear histogram for cell cycle analysis of *Lactuca sativa* root meristems. (From Coba de la Peña and Sánchez-Moreiras 2001)

blade, into a nuclear buffer. This suspension is filtered through nylon filters 30 μm pore size). If an intercalating dye is used, RNase treatment is necessary, previous to dye addition. Then, a nucleic acid-specific dye, like Ethidium Bromide (EtBr) for instance, is added for nuclei labeling. After an incubation of 30 min, labeled plant nuclei suspension is analyzed in a flow cytometer.

Of course, a mixture of nuclei in different cell cycle (active or quiescent) phases is present in this asynchronous suspension. There is a lineal correlation between fluorescence intensity of EtBr-labeled nuclei and DNA content.

One example of monoparametric histogram obtained from a labeled nuclei suspension from root meristems of *Lactuca sativa* by flow cytometry is shown in Fig. 12.1. Both axes are linear scales. Relative fluorescence intensity (proportional to the DNA content, in the X-axis of up to 1024 channels) is represented versus the number of analyzed nuclei ('Events' in the Y-axis).

In this simple case, three nuclear populations are shown:

- The first one (G0+G1) corresponds to 2C nuclei, and it includes quiescent G0, $G1_Q$ and proliferating G1 undifferentiated cells, and also 2C differentiated cells. We cannot distinguish among these different types of nuclei on the only basis of this monoparametric DNA content-depending fluorescence analysis, and all of them are placed in the same peak.
- The second peak (G2) corresponds to 4C nuclei, and it includes G2 cycling nuclei that have finished DNA replication, but also quiescent $G2_Q$ nuclei nuclei and differentiated 4C cells, not involved in the proliferating cell cycle. The mean fluorescence intensity of this 4C peak is approximately double than that of 2C peak. Usually, fluorescence intensity ratio 4C/2C is not 2, but 1.8 or 1.9, owing to labeling irregularities due to differences in chromatin condensation state (Galbraith 1989).

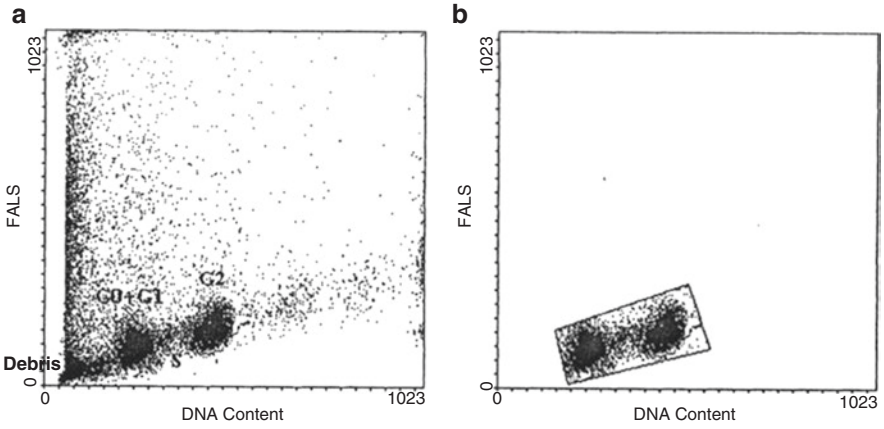

Fig. 12.2 Bi-parametric cytogram where Forward Angle Light Scatter (FALS corresponding to particle size) is represented *versus* EtBr-fluorescence intensity (corresponding to DNA content). (**a**) Total events, including the three interesting nuclei populations (G0+G1, S and G2), and population debris. (**b**) The three populations referred above are selected using gates, and only these selected nuclei are taken into account for monoparametric histogram display and analysis. (From Coba de la Peña and Sánchez-Moreiras 2001)

- Finally, the third population (S, between both peaks) is recorded as a strip connecting the first and the second peak population. This population is constituted by S-phase nuclei, in different stages of DNA replication. This is the reason of the strip shape of this population in the flow cytometry histogram. These nuclei include, of course, cycling S and non-cycling S_Q cells.

In fact, the very first analysis of nuclei suspension that must be performed by flow cytometry is a biparametric one: nuclei and debris are identified recording simultaneously EtBr-specific fluorescence intensity and particle size (FALS), as it is shown in Fig. 12.2.

In this cytogram, several principal nuclei populations are clearly identified: G0+G1 corresponds to 2C nuclei. G2 corresponds to 4C nuclei, and their size and fluorescence intensity are approximately the double than in the case of G0+G1 population. A little S population is placed between. Another population has small size and weak fluorescence intensity, and it corresponds to cellular and nuclear debris (broken nuclei, cell and membrane fragments, etc., weakly labeled with EtBr). This debris can be gated and eliminated, using discriminating windows of the cytometer software (Fig. 12.2b). The histogram showed in Fig. 12.1 results from gating and projecting the EtBr-fluorescence intensity parameter from Fig. 12.2, where debris has yet been virtually removed.

When mitosis takes place, nuclear envelope disappears, and the dispersed chromosomes (of different sizes and weak fluorescence intensities) cannot be detected or distinguished from debris in this experimental approach. Thereby, mitotic cells are lost and not detected by flow cytometry in these conditions, and this population (M) is not recorded in the histograms. In fact, for a correct evaluation of G2, M and

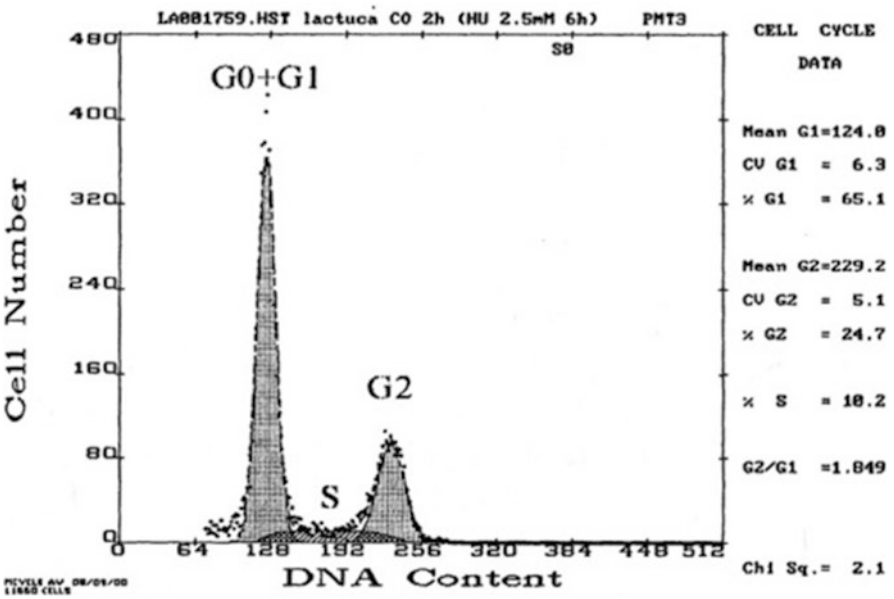

Fig. 12.3 Histogram resulting from application of computer program Multicycle (Flow System, San Diego) on raw data histogram shown in Fig. 12.1. (From Coba de la Peña and Sánchez-Moreiras 2001)

G1 lengths, mitotic indices (percent of mitosis) must be evaluated complementarily to flow cytometry using other techniques, as it is exposed in chapter about mitotic index.

This type of monoparametric analysis by flow cytometry allows simple and useful cell cycle analysis, as it will be shown below.

Usually, it is necessary to apply specific software to the flow cytometry-obtained histograms to perform a suitable cell cycle analysis from the raw data of the initial histograms. Several programs are commercially available, and each one uses different algorithms (Gray et al. 1990). These programs allow a suitable estimation of peak shape, CV of each nuclei population, %G1, %S, %G2, background subtraction, and chi-square (χ^2) estimation of fitting between raw data and estimated data. In our laboratory, the computer program Multicycle (Phoenix Flow Systems, San Diego) is used. Figure 12.3 shows Multicycle estimation of the monoparametric histogram shown above (Fig. 12.1).

Asynchronous cell populations from different tissues, meristems, and cell suspensions can be analyzed, so the percentage of cells in each cell cycle phase can be estimated. But if we are interested in obtaining metaphase chromosomes (for ulterior sorting and characterization), or in testing the effect of putative cell cycle modulators, a previous synchronization is required.

There are some commercially available inhibitors that block or stop the cell cycle in a specific phase (Planchais et al. 2000 and references therein). To avoid

malformations in the following phases, the inhibitor has to be rapidly efficient at low concentrations (Planchais et al. 2000). Cyclin-dependent kinase (CDK) inhibitors, commonly used in plant cells, are olomoucine, which inhibits at G1 to S and G2 to M transitions (Glab et al. 1994), and roscovitine and bohemine, which has been found to block the cell cycle in G1, G1/S, and G2/M in tobacco cell suspensions (Planchais et al. 1997). DNA synthesis inhibitors commonly used in plant preparations are hydroxyurea (HU) and aphidicolin. Aphidicolin causes a specific and reversible inhibition of the DNA polymerase α, leading to a removable cell cycle block at the G1/S transition (Cuq et al. 1995 and references therein), while Hydroxyurea (HU) reversibly inhibits the enzyme ribonucleotide reductase, and therefore the production of deoxyribonucleotides. Treatment with this inhibitor induces the accumulation of cells in G1 and early S phase (Doležel et al. 1999 and references therein). Anti-tubulin drugs (colchicine, oryzalin, propyzamide, etc.) and proteasome inhibitors (MG132, lactacystin) are also used to block cells at early and late mitosis (Planchais et al. 2000). Finally, starvation and physical methods have also been used for inducing partial cell cycle synchronization, principally in cell suspensions, but chemicals are more specific tool.

Once the commercial inhibitor is added, cycling cells continue the cell cycle progression up to the cycle phase point where that inhibitor has a specific effect, and all the cells will arrest the cell cycle at that phase after an incubation time. After some time, inhibitor is removed from the medium by washing and whole cycling cell population re-starts and goes on the cycle simultaneously, and this synchronous cell population progression can be acutely analyzed. In the same way, the specific effect of a putative cell cycle modulator under study can be finely analyzed. By adding the tested substance at different times after inhibitor removing, cell cycle phase and subphase-specific effects can be detected. For example, monocerin (benzopyran toxin produced by the fungus *Exserophilum turcicum*) induces a delay in the cell cycle progression of synchronized root meristems of maize, specifically in S and G2 phases, as it was revealed in a study where synchronization was performed with aphidicolin (Cuq et al. 1995). Lee et al. (1996) used hydroxyurea for root tip synchronization and subsequent metaphase chromosome isolation from maize, and Sánchez-Moreiras et al. (2008) used also this inhibitor for the first time to block cell cycle in G1 phase and analyze the effects of the secondary metabolite 2-benzoxazolinone on *Lactuca sativa* root meristems. After inhibitor removal, a synchronous cell population of about 25% of total recorded nuclei was detected in progression through S and G2 phases (%S was about 10–12% in asynchronous lettuce meristems; Sánchez-Moreiras et al. 2008).

In synchronized plant cell cultures, S nuclei can represent more than 50% of total population. At present, we are testing the putative effects of some allelochemicals on this synchronized cell cycle. Figure 12.4 shows some steps of synchronous cell cycle progression at different times after HU removal, showing both raw flow cytometry histograms (left) and the corresponding Multicycle-treated data (right). Immediately after HU inhibitor removal, 79% of detected nuclei were at G0+G1 phase, 8.4% in G2 phase, and 12.4% in S phase (Fig. 12.4a). HU has induced blockage and accumulation of nuclei in G1 phase. Start and advance of synchronized

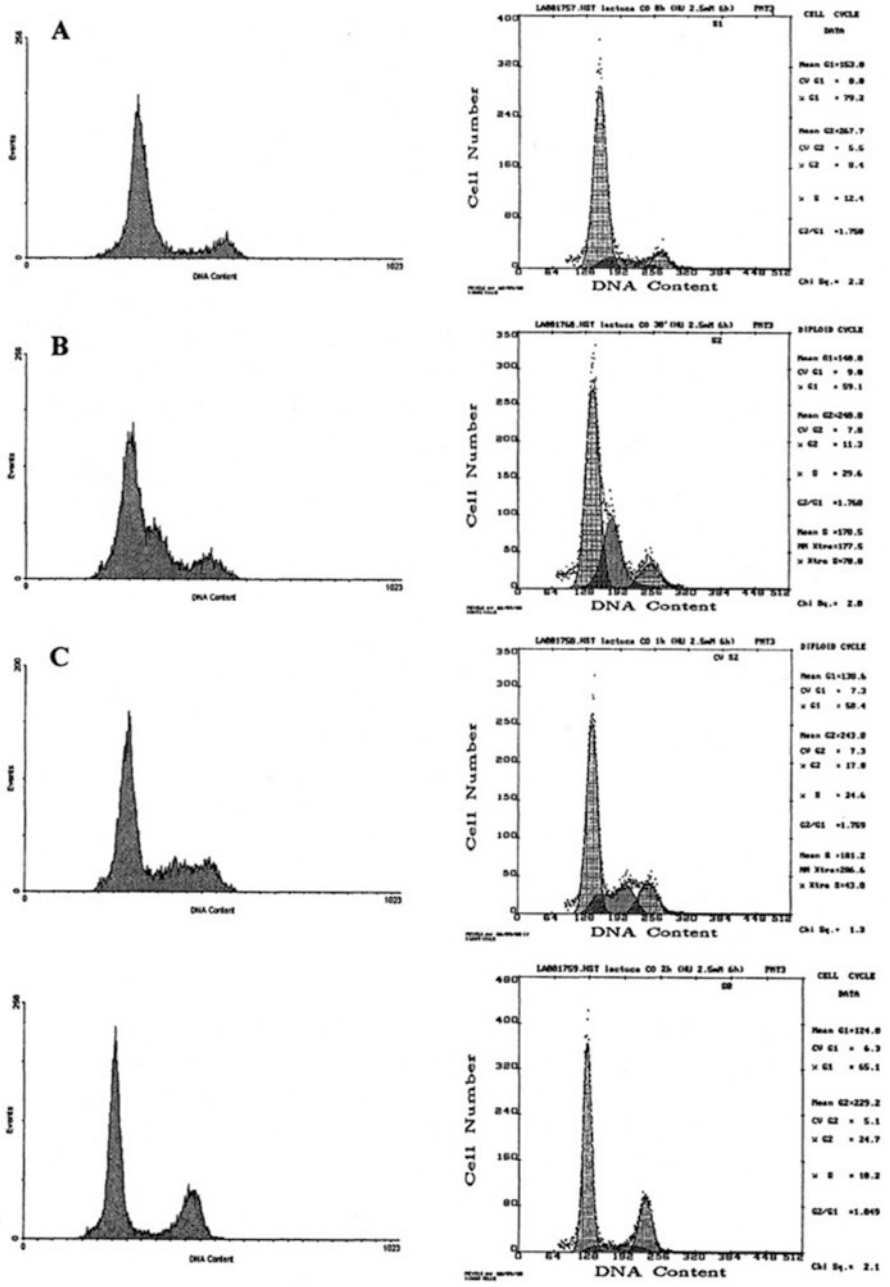

Fig. 12.4 Comparative cell cycle analysis after release of hydroxyurea-synchronized root cell meristems. (From Coba de la Peña and Sánchez-Moreiras 2001)

nuclei in S phase (29.6% of detected nuclei) is observed 30 min after (Fig. 12.4b). One hour after HU release, synchronized nuclei population begins to incorporate into G2 phase (Fig. 12.4c). Finally, all synchronized nuclei are incorporated in G2 phase of the cell cycle 2 h after HU release (Fig. 12.4d). G2 population, normally about 10% in non-synchronized meristems, reaches 24.7% in this situation. After this step, synchronized nuclei entries into mitosis and the samples have abundant metaphasic chromosomes. In this particular experimental system, a new synchronized G1 phase is not observed.

3 BI-OR Multiparametric Analysis of the Cell Cycle

Cell cycle analysis by flow cytometry can be performed measuring simultaneously other parameters, like RNA, protein contents and a wide range of antigens, using several fluorescent probes and fluorescent-labeled monoclonal antibodies. These measures allow a fine characterization and discrimination between cycling and non-cycling cells. Cell cycle can be analyzed after 5-bromodeoxyuridine (BrdUrd) incorporation. This is a thymidine analogue that is incorporated in the DNA of S-phase cells. Incorporation of Hoechst 33258 (HO), an AT-binding dye, will be reduced upon the degree of BrdUrd incorporation, owing to Hoechst do not bind DNA if BrdUrd is present instead of thymidine. Propidium Iodide (PI), an intercalating dye that is not affected by BrdUrd incorporation, is also added in this system. Simultaneous analysis of both fluorescent intensities will provide information on relative DNA content (PI) and relative fluorescence quenching (loss of HO intensity) due to DNA synthesis in presence of BrdUrd. Biparametric histograms are analyzed. By this way, it is possible to distinguish quiescent from proliferating cells, and to estimate the number of cycles they have progressed. Alternatively, anti-BrdUrd monoclonal antibodies can be used (Coba de la Peña and Brown 2001; Kim and Sederstrom 2015).

RNA levels can be detected and analyzed simultaneously to DNA (Bergounioux et al. 1988) using Acridine Orange. This is a metachromatic dye that stains differentially doubled stranded from single stranded nucleic acids. Acridine Orange fluoresces green in the first case, and red in the second, when excited in blue light (Grunwald 1993). The resulting DNA-RNA biparametric histograms allow identifying $G1_Q$, S_Q, $G2_Q$, $G1_T$, S_T, and $G2_T$ populations. Total cellular proteins can be estimated by flow cytometry using Sulphorhodamine 101 (SR 101) or Fluorescent Isothiocyanate (FITC). Moreover, a wide variety of fluorescent-labeled monoclonal antibodies against cellular antigens are available, and they can be used simultaneously with DNA-specific fluorescent dyes (Petit et al. 1993). However, as DNA denaturation is required for BrdU detection, and most protein epitopes can be destroyed avoiding classical antibody staining techniques for multiplex analysis, a novel method that overcomes DNA denaturation but still allows detection of BrdU has been developed by Cappella et al. (2008). This new procedure is based on Click chemistry detection of the thymidine analog 5-Ethynyl-2′-deoxyuridine (EdU) and

increases the options for analyzing cell cycle by flow cytometry (O'Donnell et al. 2013). This can be performed in fixed (or even living) cells or protoplasts. DNA-binding vital fluorescent dyes have been recently developed (Haugland 1996).

Thereby, DNA, RNA, total protein, and even other parameters, can be estimated simultaneously in a flow cytometer, using simultaneously several fluorescent probes, several detectors, up to three lasers for excitation, and multiparametric histograms. For instance, Onelli et al. (1997) have performed immune-characterization of PCNA (Proliferating Cell Nuclear Antigen) in synchronized root meristems of *Pisum sativum* by flow cytometry. An example of combination of this technique with molecular biology is shown in Segers et al. (1996), where it was observed that a cycling-dependent kinase gene is preferentially expressed during S and G phases in meristematic cells of *Arabidopsis thaliana*.

Different examples, more information and details about these experimental approaches are exposed in Bergounioux and Brown (1990), Bergounioux et al. (1992), Robinson et al. (1997) (for fresh plant tissues analyses), Suda and Trávníček (2006) (for dehydrated plant tissues), and Marie et al. (2000) (for phytoplankton analyses).

4 Protocol for Analyzing the Cell Cycle of Synchronized Lettuce Root Meristem by Flow Cytometry

Briefly, young lettuce plants are incubated with the cell inhibitor hydroxyurea (HU) for 6 h. After washing with distilled water, plants are immediately incubated with the treatment or the control. Samples (nuclear suspensions) are prepared from root meristems and analyzed by flow cytometry every 2 h during 12–14 h after HU removal, comparing the synchronized-cell cycle progression of treated plants with that of corresponding controls. By this way, partial or total inhibition of cell cycle can be detected (Sánchez-Moreiras et al. 2008).

A schematic representation of a general protocol for sample preparation and cell cycle analysis by flow cytometry can be shown in Fig. 12.5.

4.1 Equipment and reagents

- Flow cytometer with VIS (visible) excitation source
- Seeds of *Lactuca sativa* cv. Great Lakes, California (Fitó, S.A.) Hydroxyurea (Sigma H 8627, 2.5 mM in water, pH 6.0)
- Galbraith nuclear buffer: 45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS pH 7.0, 0.1% (w/v) Triton X-100, supplemented with 100% beta-mercaptoethanol and Tween 20.
- Ehidium Bromide (Sigma product E 8751, stock 10 mg mL^{-1} in water)

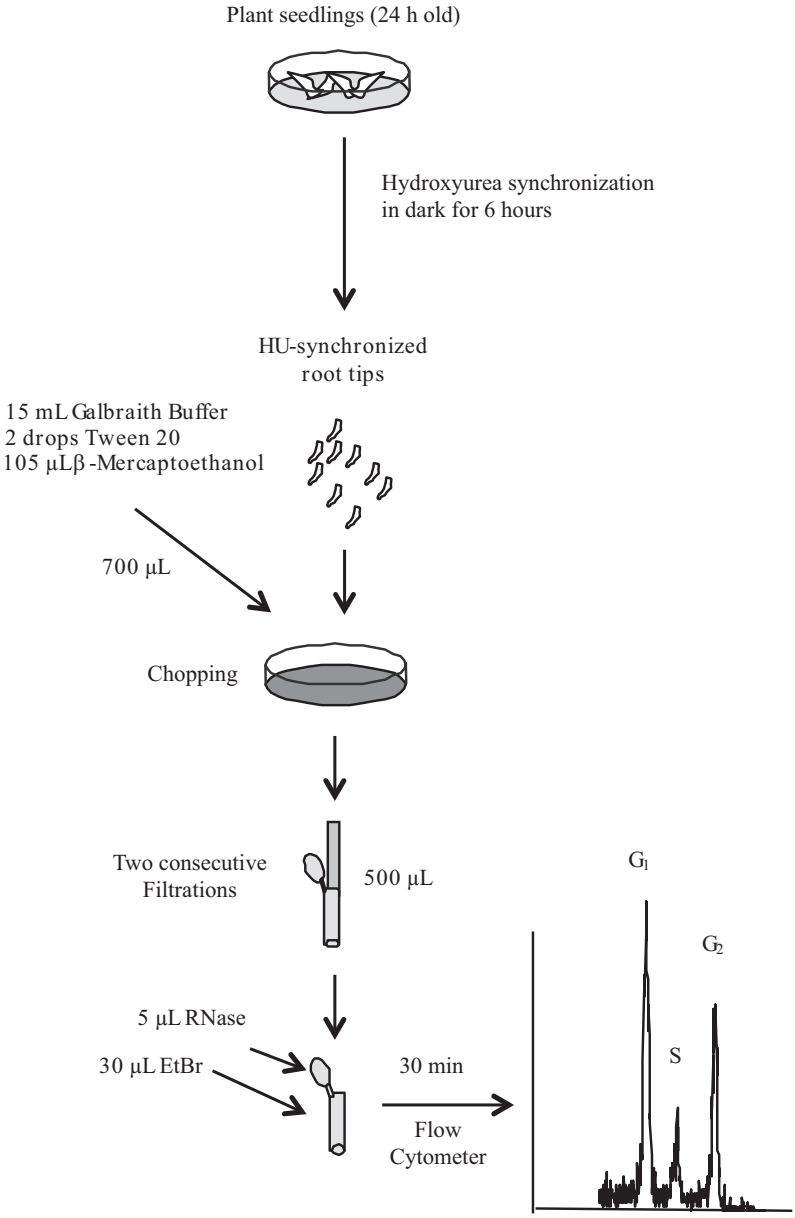

Fig. 12.5 Schematic representation of sample preparation and cell cycle analysis procedure by flow cytometry. (From Coba de la Peña and Sánchez-Moreiras 2001)

- RNase A (Boheringer Mannheim 85340024-78, stock 1% solution in Tris-HCl, NaCl and glycerol, pH 7.6)
- Heat chamber with a fixed temperature of 26 °C
- Petri dishes
- Razor blades
- 30 μm diameter nylon filters
- Micropipettes
- Plastic trays

4.2 Method

1. *Lactuca sativa* seeds are placed on moistened filter paper, into plastic trays covered with cooking foil. Seeds are germinated at 27 °C and dark for 20 h.
2. 1–3 mm-root length plants are transferred to Petri dishes containing filter paper that has been moistened with 5 mL of 2.5 mM hydroxyurea, pH 6.0. Twenty plants are placed in each Petri dish and incubated for 6 h at 27 °C in the dark.
3. HU is removed by washing twice with distilled water pH 6.0. Immediately after, plants are transferred to other Petri dishes with filter papers that have been moistened with 4 mL of either treatment (treated plants) or distilled water pH 6.0 (control plants). These plants are incubated at 27 °C and dark.
4. From this moment, and every 2 h, samples of treated plants and corresponding controls are processed simultaneously for flow cytometry analysis. The 1 mm-apical tips of root meristems from forty treated plants (that is, the content of two Petri dishes) are chopped with a razor blade on another Petri dish containing 700 μL of Galbraith buffer, supplemented with 100% Tween 20 (2 drops in 15 mL buffer) and 100% beta-mercaptoethanol (7 μL in 1 mL buffer). The obtained suspension is filtered twice through 30 μm-nylon filters, and 500 μL of filtered nuclei suspension are obtained into Eppendorf tubes. Control plants must be submitted simultaneously to the same process. The product of forty meristems constitutes one sample for flow cytometry.
5. 5 μL of 1% RNase solution are added to the nuclei suspension and, immediately after, 30 μL of 10 mg/ml Ethidium Bromide (EtBr) are added. Incubation with EtBr is for 30 min at room temperature and dark.
6. Set flow cytometer with the laser turned on 488 nm excitation wavelength. Five types of histograms (or cytograms) must be displayed in the cytometer screen:
   (a) FALS versus DNA-specific fluorescence (biparametric, see Fig. 12.3): it allows to gate debris and to eliminate it from analysis.
   (b) Peak signal versus integral signal of DNA-specific fluorescence (biparametric): it allows discarding between single nuclei and doublets.
   (c) Red signal (chlorophyll) versus yellow signal (EtBr-labeled DNA): it allows discarding stained nuclei from pigments and debris with red fluorescence.

(d) DNA fluorescence in log scale (monoparametric): it allows visualizing all peak populations.
(e) DNA fluorescence in linear scale (monoparametric, see Fig. 12.1): these are the data for cell cycle analysis.

7. Cell cycle histograms are recorded for treated and control plants every 2 h, up to arrive to 12 or 14 h of monitoring. At least 10,000 nuclei from each sample must be analyzed in the flow cytometer.
8. Data processing begins: clean histograms on a linear scale are obtained by previous gating on the other histograms.
9. Histogram profiles are analyzed using the computer program Multicycle (Flow Systems, San Diego), and G0+G1, S and G2 populations are estimated comparatively in control and BOA-treated plants.

References

Barow M, Jovtchev G (2007) Endopolyploidy in plants and its analysis by flow cytometry. In: Doležel J, Greilhuber J, Suda J (eds) Flow cytometry with plant cells. Wiley-VCH Verlag GmbH, Weinheim, pp 349–372

Bergounioux C, Brown SC (1990) Plant cell cycle analysis with isolated nuclei. Methods Cell Biol 33:563–573

Bergounioux C, Perennes C, Brown SC, Sarda C, Gadal P (1988) Nuclear RNA quantification in protoplast cell cycle phases. Cytometry 9:84–87

Bergounioux C, Brown SC, Petit P (1992) Flow cytometry and plant protoplast cell biology. Physiol Plant 85:374–386

Boyer JS (1982) Plant productivity and environment. Science 218:443–448

Cappella P, Gasparri F, Pulici M, Moll J (2008) A novel method based on click chemistry, which overcomes limitations of cell cycle analysis by classical determination of BrdU incorporation, allowing multiplex antibody staining. Cytometry A 73:626–636

Chauhan LKS, Saxena PN, Gupta SK (1999) Cytogenetic effects of cypermethrin and fenvalerate on the root meristem cells of *Allium cepa*. Environ Exp Bot 42:181–189

Chiatante D, Rocco M, Maiuro L, Scippa GS, Di Martino C, Bryant JA (1997) Cell division and DNA topoisomerase I activity in root meristems of pea seedlings during water stress. Plant Biosyst 131:163–173

Coba de la Peña T, Brown S (2001) Flow cytometry. In: Hawes C, Satiat-Jeunemaitre B (eds) Plant cell biology: a practical approach. Oxford University Press, Oxford

Coba de la Peña T, Sánchez-Moreiras AM (2001) Flow cytometry: cell cycle. In: Reigosa MJ (ed) Handbook of plant ecophysiology techniques. Kluwer Academic Publishers, The Netherlands, pp 65–80

Cookson SJ, Granier C (2006) A dynamic analysis of the shade-induced plasticity in *Arabidopsis thaliana* rosette leaf development reveals new components of the shade-adaptative response. Ann Bot 97:443–452

Cuq F, Brown SC, Petitprez M, Alibert G (1995) Effects of monocerin on cell cycle progression in maize root meristems synchronised with aphidicolin. Plant Cell Rep 15:138–142

Dayan FE, Hernández A, Allen SN, Moraes RM, Vroman JA, Avery MA, Duke SO (1999) Comparative phytotoxicity of artemisinin and several sesquiterpene analogues. Phytochemistry 50:607–614

Doležel J, Číhalíková J, Weiserová J, Lucretti S (1999) Cell cycle synchronisation in plant root meristems. Methods Cell Sci 21:95–107

Francis D (2009) What's new in the plant cell cycle? In: Lüttge U, Beyschlag W, Büdel B, Francis D (eds) Progress in botany. Springer-Verlag, Berlin, pp 33–49
Freeman JL, Rayburn AL (2006) Aquatic herbicides and herbicide contaminants: in vitro cytotoxicity and cell-cycle analysis. Environ Toxicol 21:256–263
Galbraith DW (1989) Analysis of higher plants by flow cytometry and cell sorting. Int Rev Cytol 116:165–228
Glab N, Labidi B, Qin L-X, Trehin C, Bergounioux C, Meijer L (1994) Olomoucine, an inhibitor of the cdc2/cdk2 kinases activity, blocks plant cells at the G1 to S and G2 to M cell cycle transitions. FEBS Lett 353:207–211
Graña E, Sotelo T, Díaz-Tielas C, Araniti F, Krasuska U, Bogatek R, Reigosa MJ, Sánchez-Moreiras AM (2013) Citral induces auxin-mediated malformations and arrests cell division in *Arabidopsis thaliana* roots. J Chem Ecol 39:271–282
Granier C, Cookson SJ, Tardieu F (2007) Cell cycle and environmental stresses. In: Inzé D (ed) Cell cycle control and development. Blackwell Publishing Ltd, Oxford, pp 335–355
Gray JW, Dolbeare F, Pallavicini MG (1990) Quantitative cell-cycle analysis. In: Melamed MR, Lindmo T, Mendelshon ML (eds) Flow cytometry and sorting. Wiley, New York
Grunwald D (1993) Flow cytometry and RNA studies. Biol Cell 78:27–30
Haugland RP (1996) Handbook of fluorescent probes and research chemicals. Molecular Probes Inc., Eugene
Kim KH, Sederstrom JM (2015) Assaying cell cycle status using flow cytometry. Curr Protoc Mol Biol 111:28.6.1–28.6.11
Lee JH, Arumuganathan K, Kaepler SM, Kaepler HF, Papa CM (1996) Cell synchronisation and isolation of metaphase chromosomes from maize (*Zea mays* L.) root tips for flow cytometry analysis and sorting. Genome 39:697–703
Marie D, Brown SC (1993) A cytometric exercise in plant DNA histograms, with 2C values for 70 species. Biol Cell 78:41–51
Marie D, Simon N, Guillou L, Partensky F, Vaulot D (2000) DNA/RNA analysis of phytoplankton by flow cytometry. In: Robinson JP, Darzynkiewicz Z, Dobrucki J, Hyun W, Nolan J et al (eds) Current protocols in cytometry, pp 11.12.1–11.12.14
O'Donnell EA, Ernst DN, Hingorani R (2013) Multiparameter flow cytometry: advances in high resolution analysis. Immune Netw 13:43–54
Oliva A, Moraes RM, Watson SB, Duke SO, Dayan FE (2002) Aryltetralin lignans inhibit plant growth by affecting the formation of mitotic microtubular organizing centers. Pest Biochem Physiol 72:45–54
Onelli E, Citterio S, O'Connor JE, Levi M, Sgorbati S (1997) Flow cytometry and immunocharacterization with proliferating cell nuclear antigen of cycling and non-cycling cells in synchronised pea root tips. Planta 202:188–195
Packa D (1997) Cytogenetic effects of *Fusarium* mycotoxin on root tip cells of rye (*Secale cereale* L.), wheat (*Triticum aestivum* L.) and fields bean (*Vicia faba* L. var. Minor). J Appl Genet 38:259–272
Packa D (1998) Potential genotoxicity of *Fusarium* mycotoxins in *Vicia* and *Pisum* cytogenetic tests. J Appl Genet 39:171–192
Petit JM, Denis-Gay M, Ratinaud MH (1993) Assessment of fluorochromes for cellular structure and function studies by flow cytometry. Biol Cell 78:1–13
Planchais S, Glab N, Tréhin C, Perennes C, Bureau J-M, Meijer L, Bergounioux C (1997) Roscovitine, a novel cyclin-dependent kinase inhibitor, characterizes restriction point and G2/M transition in tobacco BY-2 cell suspension. Plant J 12:191–202
Planchais S, Glab N, Inzé D, Bergounioux C (2000) Chemical inhibitors: a tool for plant cell cycle studies. FEBS Lett 476:78–83
Robinson JP, Darzynkiewicz Z, Dean PN, Hibbs AR, Orfao A, Rabinovitch PS, Wheeless LL, Galbraith DW, Lambert GM, Macas J, Dolezel J (1997) Analysis of nuclear DNA content and ploidy in higher plants. Curr Protoc Cytom 2(1):7.6.1–7.6.22

Sánchez-Moreiras AM, Coba de la Peña T, Reigosa MJ (2006) Cell cycle analyses for understanding growth inhibition. In: Reigosa MJ, Pedrol N, González L (eds) Allelopathy. A physiological process with ecological implications. Springer Academic Publishers, Dordrecht, pp 451–463

Sánchez-Moreiras AM, Coba de la Peña T, Reigosa MJ (2008) The natural compound benzoxazolin-2(3H)-one selectively retards cell cycle in lettuce root meristems. Phytochemistry 69:2172–2179

Schuppler U, He PH, John PCL, Munns R (1998) Effect of water stress on cell division and cell-division-cycle 2-like cell cycle kinase activity in wheat leaves. Plant Physiol 117:667–678

Scofield S, Jones A, Murray JAH (2014) The plant cell cycle in context. J Exp Bot 65:2557–2562

Segers G, Gadisseur I, Bergounioux C, De AEJ, Jacqmard A, Montagu MW, Inzé D (1996) The Arabidopsis cyclin-dependent kinase gene cdc2bAt is preferentially expressed during S and G sub (2) phases of the cell cycle. Plant J 10:601–612

Suda J, Trávníček P (2006) Reliable DNA ploidy determination in dehydrated tissues of vascular plants by DAPI flow cytometry—new prospects for plant research. Cytometry A 69A:273–280

Tardieu F, Granier C (2000) Quantitative analysis of cell division in leaves: methods, developmental patterns and effects of environmental conditions. Plant Mol Biol 43:555–567

Wisniewska H, Chelkowski J (1994) Influence of deoxynivalenol on mitosis of root tip cells of wheat seedlings. Acta Physiol 16:159–162

Wonisch A, Tausz M, Müller M, Weidner W, De Kok LJ, Grill D (1999) Treatment of young spruce shoots with SO_2 and H_2S: effects on fine root chromosomes in relation to changes in the thiol content and redox state. Water Air Soil Poll 116:423–428