4
Nuclear DNA Content Measurement

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Overview

This chapter reviews essential aspects of the flow cytometric studies of plant DNA contents, starting with a discussion of the recently updated revised terminology for presenting nuclear DNA amounts. Plants have a relatively complicated life cycle with alternation of generations and nuclear phases, they exhibit somatic polyploidization during ontogenetic differentiation and generative polyploidization during evolution. The terms ''holoploid genome size'' and ''monoploid genome size'', and their acronyms C-value and Cx-value, respectively, are promoted as elements of a precise terminology for unambiguous data presentation. DNA amounts can be presented relative to a reference species (standard) or in absolute units of picograms or base pairs, for which the correct conversion factor is specified. The methodological aspects of preparing samples for DNA content measurements are discussed with special consideration of standardization and the interfering role of secondary metabolites. Internal standardization with a plant standard is regarded as the most important approach to minimizing the effect of fluorescence inhibitors and balancing out all technical variations which occur during an experiment. It is accepted that a consensus on a set of standard species covering the whole range of C-values has still not been achieved. Some rules are outlined for assuring data quality and sufficiently detailed data presentation. As far as the methodological side of measuring DNA amounts is concerned, it is expected that important future research developments will occur in the field of preparative improvements to overcome stoichiometric errors, the utilization of dormant diaspores and conserved tissues for flow cytometry, and that a reliable plant standard species will be established in addition to guidelines for internal calibration.

4.1
Introduction

Estimation of DNA content in cell nuclei is one of the important applications of flow cytometry (FCM) in plant sciences. Although first results on plant material

(root tips of *Vicia faba*, Fabaceae) with the then novel methodology were reported in the early 1970s (Heller 1973), it was not before the introduction of the ingenious chopping method for isolation of plant nuclei by Galbraith et al. (1983), that FCM became widely accepted as a convenient approach for measuring DNA contents and genome size. Galbraith et al. (1983) circumvented cumbersome protoplasting or enzymatic isolation of nuclei by simply chopping up with a razor blade fresh leaves of tobacco (*Nicotiana tabacum*, Solanaceae) and a number of other plant species in an appropriate buffer plus detergent and then sieving out large particles, whereby enough nuclei were released to yield clear histograms upon FCM. This paper was also notable in applying internal standardization with chicken red blood cells (CRBCs) for genome size determination (although the GC-specific fluorochrome mithramycin was used for staining DNA, which overestimates DNA amount in GC-rich genomes (Doležel et al. 1992)). The DNA content of the standard was determined chemically. In those early days, the cost of instruments, which were not easy to operate, was the main reason why they were not used outside the field of biomedical sciences (cf. Chapter 1). Today, there are affordable instruments are on the market, so that even small botany laboratories are increasingly using FCM.

The advantages of flow cytometry over static cytometry are clear: speed of preparation and data gathering, and higher precision due to high numbers of nuclei measured and possibly also due to a more homogeneous staining of isolated nuclei in suspension. A mysterious disadvantage of static cytometry (i.e. mainly Feulgen densitometry), which is explained neither by notoriously small sample sizes nor by technical difficulties, is the plain fact, that many published results are unreliable for unknown and untraceable reasons (Greilhuber 2005). This is apparently not the case to a comparable extent with FCM data. An advantage of static cytometry is the absence of debris, because only nuclei are measured. Problems common to both technologies are bias caused by variation in chromatin compactness and the interference of secondary metabolites with the staining process. Presently it seems that the latter source of error is specific to plants, but, as phenolic compounds are involved and these also occur in animals (e.g. phenoloxidases play a role in melanin production), the problem may exist with zoological material as well, but remained unrecognized. There is also another particularity of FCM: the nuclei are measured without visual selection, what may be judged as being more objective than selecting nuclei in the microscope by eye. However, in critical cases light-microscopic evidence must be obtained for unequivocal interpretation of FCM results, for instance when the histogram peak of unreplicated nuclei is small and could be overlooked, or when genome size is very small and debris is abundant.

It is the purpose of the present chapter to discuss basic problems associated with FCM work on nuclear DNA content in plants. The biological significance of genome size and variation in DNA content is discussed in Chapters 5, 7, 9 and 15, and genetic aspects are covered by Chapters 6, 9, 14, 16 and 17. The first plant DNA flow cytometry database (FLOWER) is presented in Chapter 18. A particularly useful review on plant DNA flow cytometry is the publication by Doležel and Bartoš (2005).

4.2
Nuclear DNA Content: Words, Concepts and Symbols

Swift (1950) introduced the symbol "C", meaning the "constant" of DNA content, which is represented in multiples in nuclei of various tissues of an organism (see Bennett and Smith 1976; Greilhuber et al. 2005). Bennett and Smith (1976) defined the C-value (i.e. the 1C-value!) as the "DNA content of the unreplicated haploid chromosome complement". To avoid the ambiguity of terms such as "genome size" and "nuclear DNA content" or "basic nuclear DNA content" or "amount", Bennett et al. (1998) restricted "genome size" to the monoploid genome, while "C-value" continued to refer to the DNA content of the complete chromosome complement. But it was soon felt that this restricted use would entirely eliminate the established and phonetically pleasing term "genome size" from the discourse, because often the degree of polyploidy is unknown, genomic reconstructions in polyploids reshuffled ancestral genomes, and possibly all plants have experienced one or more polyploidizations in their ancestry (Wendel 2000).

Greilhuber et al. (2005) thus presented a slightly modified and complete terminology, which was guided (i) by accepting an explicit link between genomic DNA content designations and the chromosome numbers *n* (the haplophasic or meiotically reduced number) and *x* (the basic chromosome number of a polyploid series), and (ii) by striving at linguistic consistency in using full terms and their acronyms. At the same time the well-established symbol C had to remain unchanged. The term genome size thus retains its everyday meaning as a covering term usable in titles, introductory and concluding phrases. The adjectives "monoploid" and "holoploid" distinguish between genome size of the monoploid genome (= the single genome with *x* chromosomes, of which there are two per unreplicated nucleus in a diploid individual and several in a polyploid individual) and the complete, that is, holoploid genome. The respective abbreviations are C-value for the holoploid genome and Cx-value for the monoploid genome (the letter x refers to the basic chromosome number *x*). Quantitative data are given with numerical prefix, as 1C-, 2C-, 1Cx-, 2Cx-values and so on. A summary of the terminology is presented in Table 4.1.

Plants in particular are more complicated than most animals owing to their complex life cycle with alternation of generations and alternation (or not) of nuclear phases, and the frequently occurring generative and somatic polyploidy. Thus, the application of an unambiguous terminology is essential but not always adhered to in publications. This can lead to confusion.

There are basically four different kinds of DNA copy number status.

4.2.1
Replication–Division Phases

Replication–division phases of the mitotic nuclear cycle are related to its G_1, S and G_2 phases (cf. Chapter 14, Fig. 14.1). Replication and division lead to changes in DNA content expressed in terms of C. For instance, mitotically active nuclei

Table 4.1 Genome size terminology (from Greilhuber et al. 2005).

Genome status	Monoploid	Holoploid
Chromosome number designation	*x*	*n*
Covering term for genomic DNA content	Genome size	Genome size
Kinds of genome size	Monoploid genome size	Holoploid genome size
Short terms	Cx-value	C-value
Short terms quantified	1Cx, 2Cx, etc.	1C, 2C, etc.

in a haplophasic moss gametophyte cycle between 1C and 2C, in a diplophasic angiosperm root tip between 2C and 4C, and in a triploid endosperm between 3C and 6C.

4.2.2 Alternation of Nuclear Phases

Alternation of nuclear phases (not to be confused with alternation of generations!) is associated with meiotic reduction and fertilization (in angiosperms including endosperm fertilization). The nuclear phase status is denoted using the letter *n*. *n* indicates the meiotically reduced, haplophasic chromosome number, 2*n* the unreduced, diplophasic number, and 3*n*, 5*n*, and so forth the endospermic chromosome numbers. The DNA content levels are indicated using the letter C, 1C usually being the lowest level recognized, such as in an unreplicated nucleus in a haploid moss gametophyte, or a sperm nucleus of an animal. 1C levels can also be calculated from higher C-levels by dividing the DNA amount by the corresponding ploidy level. Thus, it is not necessary to measure haplophasic unreplicated nuclei to determine a 1C-value of a seed plant.

4.2.3 Generative Polyploidy Levels

Generative polyploidy levels refer to the presence of one, two, or more monoploid genomes (each with chromosome number *x*) in the complete, holoploid genome with chromosome number *n* (Greilhuber et al. 2005), which characterize single individuals, populations or taxa. The level of generative polyploidy is indicated by the letter *x*. A diploid angiosperm species has $2n = 2x$, a tetraploid $2n = 4x$, and so on. But note, that a plant of a haploid moss species has $n = x$ while a plant of a diploid species has $n = 2x$ (the haplophase dominates; see Chapter 12). A symbol was needed for presenting not only C-values, but also the amounts of DNA in the monoploid genomes involved and their multiples. Consequently, Cx was introduced, 1Cx being the amount of DNA of an unreplicated monoploid genome (see above and Table 4.1; Greilhuber et al. 2005). Cx-values will usually

be average values unless the monoploid genomes constituting a holoploid genome can be measured separately.

4.2.4 Somatic Polyploidy

Somatic polyploidy is caused by endocycles of replication or by mitotic restitution (breakdown of mitosis in various stages) in somatic tissues (compare Chapter 15). The degree of polyploidy and the amount of DNA in such nuclei can be given as C-levels. It would be misleading here to present DNA amounts on the basis of n, because this denotes a chromosome number, and chromosomes can be unreplicated or replicated. For example, an endopolyploid root cell interphase nucleus in *Arabidopsis thaliana* (Brassicaceae) with 1C = 0.16 pg or 157 Mbp ($n = 5$, $2n = 10$) with a DNA content (not genome size!) of 2.56 pg is in 16C. From this value it is not evident, whether the nucleus is octoploid or 16-ploid. However, microscopically a spontaneous mitotic telophase nucleus with 80 chromatids and in 16C can be termed 16-ploid, while the preceding prophase nucleus in 32C would have shown 80 prophase chromosomes, thus being also 16-ploid. For comparative purposes it is possible to indicate the number of (endo)reduplication rounds to reach a certain C-level, as Barow and Meister (2003) used it for comparing different tissues in a number of angiosperm species, that is, 2C nuclei receive cycle value 0, 4C receive value 1, 8C receive value 2, and so forth. For tissues and plant organs averaged cycle values can so be given.

These rules have not only theoretical but also practical significance, for example, in labeling histograms of DNA content. A diagrammatic example of how flow histograms of different cytotypes would be labeled is presented in Fig. 4.1. In Chapter 6, Fig. 6.3, the Cx symbol is used to label histogram peaks in the flow cytometric seed screen of mixed samples of tetraploid *Hypericum perforatum* (Hypericaceae). Previously, Śliwińska and Lukaszewska (2005) analyzed polysomaty in di-, tri- and tetraploid sugarbeet, and labeled the G_1 peaks 2C, 3C and 4C, respectively, the G_2 peaks 4C, 6C, and 8C, respectively, and so on. Now that the Cx symbol is available, it is not advisable to label the G_1 peaks of di-, tri- and tetraploid individuals of a higher plant species as 2C, 3C and 4C, because all are in 2C. But it is correct to label these peaks with 2Cx, 3Cx and 4Cx (compare Fig. 4.1). The G_2 peaks of these plants would be correctly labelled 4Cx, 6Cx and 8Cx, and so on. Any individual of zygotic origin starts at 2C, be it diploid, triploid or whatever, because it starts at 2n. This avoids an infinite progression in C-levels with the advent of higher levels of generative polyploidy. For indicating these, x and Cx exist. Likewise, haplophasic individuals such as haplophasic sporophytes and gametophytes start at 1C, notwithstanding that in some cytogenetic traditions (not followed here) haplophasic sporophytes and animals such as male hymenoptera are given the chromosome number $2n$ (cf. John 1990).

Schween et al. (2003) used the C and G symbols in combination to indicate DNA amounts in the moss *Physcomitrella* (Funariaceae), so that the 1C peak was

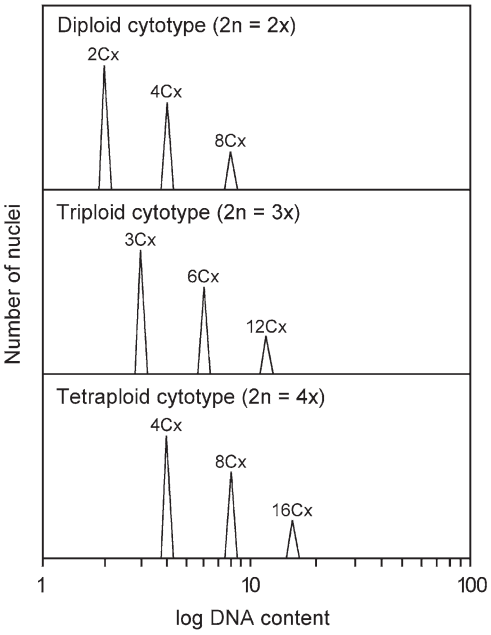

Fig. 4.1 Diagrammatic sketch of labeling peaks on DNA content histograms of cytotypes of different ploidy using the C/Cx-terminology to describe nuclear DNA contents (Greilhuber et al. 2005). Note that in each cytotype the first peak is to be regarded the 2C-peak of that cytotype. For further explanation see text.

identified as "$1CG_1$", the 2C peak "$1CG_2$", and the 4C peak "$2CG_2$ or $4CG_1$". Here, C was obviously used in the sense of *n*, which should be avoided (see above).

4.3 Units for Presenting DNA Amounts and their Conversion Factors

Nuclear DNA amounts can be presented relative to the DNA content of biological standard nuclei (%, ratio), as mass units (usually picograms, pg), or as number of base pairs (bp, Mbp, Gbp). Although pg have long been used as the preferred units, with photometric methods mass is measured indirectly at best. Rather it is the relative number of base pairs, which is estimated, provided the DNA stain binds stoichiometrically and without base-dependent bias. Therefore, more recently the prevailing convention for presenting the amount of DNA is by specifying the number of base pairs. It should be noted that molecular biologists often use base number (kb, Mb, Gb) instead of base pair number, meaning DNA length instead of mass. As DNA is a double-stranded molecule, a misunderstanding can

cause a two-fold error in calculating DNA content. Thomas et al. (2001) made this mistake when calculating the size of the human genome; however the error was corrected by Doležel et al. (2003). Presenting DNA amounts as the number of base pairs (bp) rather than bases is unequivocal and is therefore recommended.

Surprisingly enough, partially incorrect or poorly-supported conversion factors for pg into bp number and vice versa have been used for a long time and are even being used today. A factor of 0.965×10^9 to convert pg into base pair number has been in use until recently (Bennett and Smith 1976) with reference to Straus (1971), who reported "5.8 pg or 5.6×10^9 nucleotide pairs" for the frog, *Rana pipiens*, but did not give a conversion factor. Cavalier-Smith (1985, Preface, p. x) presented (without a derivation) a correct factor of 0.98×10^9, which was rounded up to the second decimal place. A derivation of the factor has been published recently (Doležel et al. 2003), which is as follows:

$$\text{DNA content (bp)} = (0.978 \times 10^9) \times \text{DNA content (pg)}$$

$$\text{DNA content (pg)} = \text{DNA content (bp)}/(0.978 \times 10^9)$$

Table 4.2 gives the relative weights of nucleotide pairs, AT = 615.3830 and GC = 616.3711, whereby the loss of one H_2O molecule during the formation of one phosphodiester linkage is taken into account. Note, that GC differs from AT only 1.0016-fold in weight, so that negligible bias is introduced in using mass units instead of base pair number. At physiological pH the proton is dissociated from the phosphate of any nucleotide. Assuming a 1:1 ratio of AT to GC and disregarding modified nucleotides, the mean molecular weight of one nucleotide pair is 615.8771. Multiplying the relative molecular weight by the atomic mass unit 1u, which equals 1/12 of a mass of ^{12}C, that is, 1.660539×10^{-27} kg, the mean weight of one nucleotide pair can be calculated to be 1.023×10^{-9} pg. 1 pg of DNA thus represents 0.978×10^9 base pairs.

Table 4.2 Relative molecular weights of nucleotides.

Nucleotide	Chemical formula	Relative molecular weight
2′-deoxyadenosine 5′-monophosphate	$C_{10}H_{14}N_5O_6P$	331.2213
2′-deoxythymidine 5′-monophosphate	$C_{10}H_{15}N_2O_8P$	322.2079
2′-deoxyguanosine 5′-monophosphate	$C_{10}H_{14}N_5O_7P$	347.2207
2′-deoxycytidine 5′-monophosphate	$C_9H_{14}N_3O_7P$	307.1966

Calculated with the following standard atomic weights: $A_r(H) = 1.0079$, $A_r(C) = 12.0107$, $A_r(N) = 14.0067$, $A_r(O) = 15.9994$, $A_r(P) = 30.9738$. Standard atomic weights are scaled to nuclide ^{12}C with $A_r(^{12}C) = 12$ and rounded to four decimals. (From Doležel et al. 2003).

4.4 Sample Preparation for Flow Cytometric DNA Measurement

4.4.1 Selection of the Tissue

In principle, every tissue containing vital nuclei should be suitable for measurement of nuclear DNA content with FCM, but the presence or absence of endogenous fluorescence inhibitor substances and coatings of debris (see below) primarily influences the quality of the results. Generally, fresh almost fully expanded leaves are preferable. Very young leaves may be less suitable because of their higher content of inhibitors. It is preferable to use colorless plant organs rather than those colored by anthocyan (a fluorescence inhibitor, see below). If results are unsatisfactory, other tissues are worth considering. The light regime during plant cultivation will influence the synthesis of flavonoids, anthocyans and other phenolics, and should be selected so as to minimize the production of these substances (see Section 4.6). This effect has unintentionally been shown by Price and Johnston (1996). Nevertheless, little is known about the effect of light during cultivation with regard to FCM, and targeted studies are required. Optimal light for plant growth may not necessarily be optimal for nuclear DNA flow cytometry.

There are several investigations indicating the suitability of dry seed material for determination of nuclear DNA content by FCM. Normal seed contains a diplophasic embryo and depending on the taxon may also contain endosperm (basically haplophasic endosperm in gymnosperms and most frequently triplophasic, but occasionally diplophasic and pentaplophasic endosperm in sexual angiosperms, and other levels in hybrid situations and in apomicts; see Chapter 6). Bino et al. (1992, 1993) followed the replication levels in germinating seeds of a number of plant species and observed triploid endosperm in dry seed of *Cichorium endivia* and *Lactuca sativa* (both Asteraceae), *Solanum melongena* and *Lycopersicon esculentum* (both Solanaceae) and *Spinacia oleracea* (Chenopodiaceae/APG: Amaranthaceae), the latter two species exhibiting only the 6C-level (Bino et al. 1993). Matzk et al. (2000, 2001, 2003, 2005) analyzed the relative nuclear DNA content in dry seeds of *Arabidopsis thaliana*, *Hypericum perforatum*, *Poa annua* (Poaceae) and other angiosperms for reproduction mode screening with considerable success (the Flow Cytometric Seed Screen, FCSS; see Chapter 6). The technical side of the approach used by Matzk is remarkable, that is, dry seeds or parts thereof are crushed between two sheets of sand-paper, rinsed off with DAPI buffer, and measured. Baranyi and Greilhuber (1996) and Baranyi et al. (1996) measured the genome size of some poorly-germinating pea accessions using ethidium bromide and hypocotyl and root samples from briefly hydrated seed. Śliwińska et al. (2005) found that hypocotyls from non-hydrated seeds of *Brassica napus* (Brassicaceae) and several other crop species gave more reliable results than leaf tissue. Thus, this approach should be widely tested for studies of genome size which require intercalating dyes. On the one hand it is surprising that chromatin from dormant tissue can be easily stained with fluorochromes.

However, on the other hand it is possible that dry cells release less nucleases into the nuclear isolation solution than turgid cells from soft tissue (Chapter 6) and that certain organs such as hypocotyl contain fewer inhibitors or that dry tissues release less of them into solution (see Section 4.6). For optimal results it seems to be essential to first crush the dry tissue and then to immediately stain in buffer and measure the fluorescence (see Chapter 6). This is reminiscent of the behavior of herbarium material subjected to FCM; in this case the best results were obtained by chopping up the sample in DAPI staining buffer without pre-soaking (Suda and Trávníček 2006; Chapter 5). Targeted investigations into the time scale on which such measurements can be performed with different categories of seed, are desirable. Measurements can even be done with non-germinable seeds (Chapter 6), but if so, how old should such seeds be? And what are the reasons for quality decay with respect to DNA structure? The "seminal approach" has the potential to open a new era for biodiversity-oriented genome size studies (cf. Chapter 7), but the particularities of the material (e.g. replication levels and endopolyploidy in the embryo, spontaneous hybridization, fertilization with unreduced gametes and apomixis; cf. Chapter 6) will need to be carefully considered.

4.4.2 Reagents and Solutions

Researchers involved in the early work with plant FCM isolated protoplasts with hydrolytic enzyme mixtures, lysed the protoplasts and stained them with a fluorochrome, mainly DAPI. Doležel et al. (1989) give examples of *Zea mays* (Poaceae) and *Medicago sativa* (Fabaceae) callus and leaf material.

Today, the method of preparing a suspension of nuclei for measurement follows the ingeniously simple procedure of Galbraith et al. (1983). It consists basically of (i) chopping up the plant material with a sharp razor blade to release nuclei into isolation buffer or buffer component, (ii) sieving the homogenate to remove large particles, and (iii) staining the nuclei in (buffered) suspension with the fluorochrome of choice. RNase should be added, if intercalating dyes such as ethidium bromide (EB) or propidium iodide (PI) rather than the base-specific minor grove-binding Hoechst dyes and DAPI (AT specific), or mithramycin, olivomycin and chromomycin (GC specific) are used. It is important to use PI or EB to quantify the DNA content without biasing the results with the base content (Doležel et al. 1992). A saturation curve of PI is shown in Fig. 4.2, indicating that PI concentrations between 50 and 150 mg l^{-1} are appropriate. A similar result was obtained by Loureiro et al. (2006a) for *Pisum sativum* isolated with four different buffers. The steps can be carried out in sequence or can be combined so that chopping, staining and RNase digestion are completed in one or two steps (i.e. the chopping buffer also contains the RNase, or in addition the dye). RNase addition may often show no effect due to the low RNA content, in leaves for instance, and thus may seem dispensable, but is essential with tissues rich in RNA such as meristems and seeds, and is also for principal reasons an established step in the procedure. It should be noted that chopping up the tissue in the stain solution, as

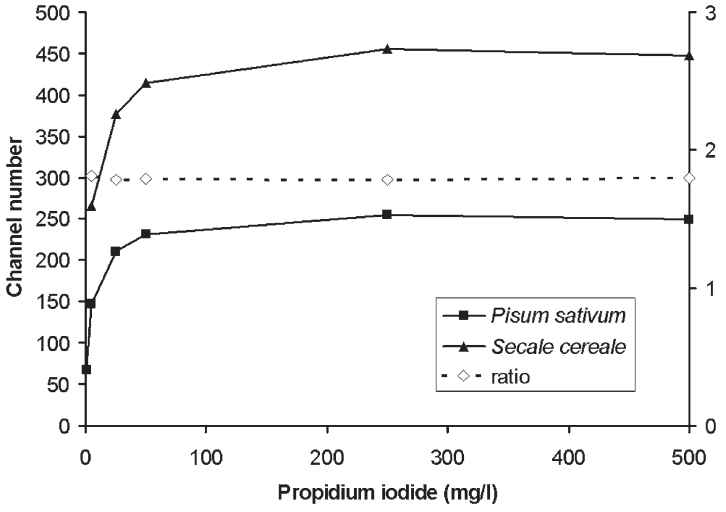

Fig. 4.2 Propidium iodide saturation curve. Nuclei were isolated from co-chopped leaves of *Pisum sativum* "Kleine Rheinländerin" and *Secale cereale* "Elect" in Otto buffer component I. The isolate was divided into 0.4-ml aliquots, which were treated with RNase at 37 °C for 30 min and immediately stored in the refrigerator. The aliquots were then stained with Otto buffer component II supplemented with 0.5, 5, 25, 50, 250 and 500 mg l^{-1} propidium iodide and measured with a flow cytometer (Partec PA II) after a 1-h incubation at 7 °C. (Original by E. M. Temsch).

is sometimes practised, increases the likelihood of skin and laboratory contamination of the sample and also increases the number of disposables that would need to be treated as toxic waste. Also RNase spills can be problematic in some laboratories. It should therefore be carefully considered whether a small gain in time outweighs laboratory safety (but note the recommendations on work with dry material, see above).

4.4.2.1 **Isolation Buffers and DNA Staining**

Various isolation buffers are used in plant FCM (Table 4.3). Staining is carried out at neutral or slightly basic pH and there is some detailed information available on the effect of pH on DNA specificity for the stain Hoechst 33258. Hilwig and Gropp (1975) showed that in cytological preparations at pH 2, nucleoli and cytoplasm, probably the RNA, are stained as well as chromatin DNA, while at pH 7 only chromatin is stained. Slides stained at pH 2 lost the non-specific DNA staining if mounted with pH 7 buffer, and did not regain it at pH 2 unless re-stained. Other proton concentrations were not tested. For DAPI even less information is available, despite its wide use in cytogenetics and its high level of biochemical evaluation (Kapuscinski 1995). In chromosome cytology, DAPI staining of DNA is generally carried out at pH 7, and this is also the case in plant FCM. However, Wen et al. (2001) in a study on dye concentration and pH in biomedical DNA measurements, found in tumor and mouse cell lines the best CVs (coeffi-

Table 4.3 Ten most popular non-commercial nuclear isolation buffers in plant DNA flow cytometry. Buffers are arranged in decreasing order of preference according to the FLOWER database (see Chapter 18).

Buffer	Composition[a]	References
Galbraith's	45 mM $MgCl_2$; 30 mM sodium citrate; 20 mM MOPS; 0.1% (v/v) Triton X-100; pH 7.0	Galbraith et al. (1983)
$MgSO_4$	9.53 mM $MgSO_4.7H_2O$; 47.67 mM KCl; 4.77 mM HEPES; 6.48 mM DTT; 0.25% (v/v) Triton X-100; pH 8.0	Arumuganathan and Earle (1991)
LB01	15 mM Tris; 2 mM Na_2EDTA; 0.5 mM spermine.4HCl; 80 mM KCl; 20 mM NaCl; 15 mM β-mercaptoethanol; 0.1% (v/v) Triton X-100; pH 7.5	Doležel et al. (1989)
Otto's[b]	Otto I: 100 mM citric acid monohydrate; 0.5% (v/v) Tween 20 (pH approx. 2–3) Otto II: 400 mM $Na_2PO_4.12H_2O$ (pH approx. 8–9)	Otto (1990), Doležel and Göhde (1995)
Tris.$MgCl_2$[c]	200 mM Tris; 4 mM $MgCl_2.6H_2O$; 0.5% (v/v) Triton X-100; pH 7.5	Pfosser et al. (1995)
Baranyi's[b]	Baranyi solution I: 100 mM citric acid monohydrate; 0.5% (v/v) Triton X-100 Baranyi solution II: 400 mM $Na_2PO_4.12H_2O$; 10 mM sodium citrate; 25 mM sodium sulfate	Baranyi and Greihuber (1995)
Bergounioux's	"Tissue culture salts" supplemented with 700 mM sorbitol; 1.0% (v/v) Triton X-100; pH 6.6	Bergounioux et al. (1986)
Rayburn's	1 mM hexylene glycol; 10 mM Tris; 10 mM $MgCl_2$; 0.5% (v/v) Triton X-100; pH 8.0	Rayburn et al. (1989)
Bino's	200 mM mannitol; 10 mM MOPS; 0.05% (v/v) Triton X-100; 10 mM KCl; 10 mM NaCl; 2.5 mM DTT; 10 mM spermine.4HCl; 2.5 mM $Na_2EDTA.2H_2O$; 0.05% (w/v) sodium azide; pH 5.8	Bino et al. (1993)
De Laat's	15 mM HEPES; 1 mM EDTA $Na_2.2H_2O$; 0.2% (v/v) Triton X-100; 80 mM KCl; 20 mM NaCl; 15 mM DTT; 0.5 mM spermine.4HCl; 300 mM sucrose; pH 7.0	de Laat and Blaas (1984)

[a] Final concentrations are given. MOPS, 4-morpholinepropane sulfonate; DTT, dithiothreitol; Tris, tris-(hydroxymethyl)-amino-methane; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(hydroxymethyl)piperazine-1-ethanesulfonic acid. For details on the buffer preparation see the original reference(s).

[b] pH of the buffers is not adjusted.

[c] The original recipe and reference for Tris.$MgCl_2$ is presented. Several minor modifications have been made so far, nonetheless, the basic composition remains stable.

cients of variation) and least debris at pH 6, while at pH 8 the histograms had already collapsed. At pH 7, in the mouse cell line MAT-B1 the histogram was still highly resolved, while in the line P388/R84 a significant decay in quality was observed. This is difficult to explain and stands in contradiction to the results of Otto et al. (1981). Studies on the effects of pH on staining intensity, histogram quality and DNA specificity in plant FCM are thus urgently required.

PI and EB stain DNA above pH 4, with some increase at higher pH as shown for EB by Le Pecq and Paoletti (1967). The buffer should also provide ionic strength for PI and EB to stain the nucleic acid quantitatively (Le Pecq and Paoletti 1967). If nuclei are isolated at acidic pH in citric acid plus detergent (Otto procedure; Otto et al. 1981), the dye must be added in basic solution (Na_2HPO_4) so that a final neutral pH is achieved (first used with unfixed plant nuclei by Doležel and Göhde (1995), then slightly modified by Baranyi and Greilhuber (1995), and later called the ''two-step procedure'' by Doležel et al. (1998)).

Isolation buffers, in addition to releasing nuclei from the cytoplasm in sufficient quantities, must also maintain nuclear integrity throughout the experiment, protect DNA from degradation by endonucleases and permit stoichiometric DNA staining. From about 26 different isolation formulas described, six are commonly used in plant DNA flow cytometry (Loureiro et al. 2006a; Table 4.3). Their usual components include: (i) organic buffer substances (e.g. Tris, MOPS and HEPES) to stabilize the pH of the solution (usually set between 7.0 and 8.0, which is compatible with common DNA fluorochromes); (ii) non-ionic detergents (e.g. Triton X-100, Tween 20) to release and clean nuclei, and decrease the aggregation affinity of nuclei and debris (note that ionic detergents such as sodium dodecyl sulfate would change the fluorescence properties of the dye molecule; Kapuscinski 1995); (iii) chromatin stabilizers (e.g. $MgCl_2$, $MgSO_4$, spermine); (iv) chelating agents (e.g. EDTA, sodium citrate) to bind divalent cations, which serve as nuclease cofactors; and (v) inorganic salts (e.g. KCl, NaCl) to achieve proper ionic strength (Doležel and Bartoš 2005).

''Otto's buffer'', which is in fact the well-known McIlvaine's buffer system (e.g. Rauen 1964, pp. 92, 95) plus detergent, was first introduced to FCM in combination with DAPI by Otto et al. (1981) for ethanol-fixed mouse cells, which were resuspended in 0.2 M citric acid plus 0.5% Tween 20, adjusted to pH 7.4 and stained. With regard to this technique Otto et al. (1981) refer to Pinaev et al. (1979), who isolated non-fixed HeLa chromosomes in 0.1 M citric acid plus 0.1 M sucrose plus 0.5% Tween 20. Ulrich and Ulrich (1991) used Otto's buffer for nuclei isolation from living plant tissue, but fixed the nuclei in acetic ethanol; staining and analysis was again carried out in Otto's buffer with very narrow CVs obtained. Otto's buffer system plus DAPI was first used for unfixed plant nuclei by Doležel and Göhde (1995) for sex identification in *Melandrium* (Caryophyllaceae) and basically (with minor modification) also by Baranyi and Greilhuber (1995) to demonstrate the lack of variance of genome size in *Pisum sativum* (Fabaceae). This buffer system was obviously the essence of a commercial Partec buffer (solutions A and B) with proprietary composition in the early 1990s. It consists of two components, citric acid plus detergent (''Otto I'') for nuclei isolation, and the

basic Na_2HPO_4 plus fluorochrome ("Otto II"), which is added to the isolate for staining at neutral pH. Baranyi and Greilhuber (1996) first modified and applied this system for EB and PI staining (with some non-essential additions; J. Greilhuber and E. M. Temsch, unpublished data). Otto's buffer differs essentially from other buffers, because the first step combines isolation of nuclei with mild fixation and possibly some histone removal.

The other buffers (Table 4.3) work *a priori* at near-neutral pH and are based on popular organic buffer substances such as MOPS (Galbraith et al. 1983), Tris (Doležel et al. 1989; Pfosser et al. 1995) and HEPES (Arumuganathan and Earle 1991). With these buffers it is intended to keep the nuclei in an intact or even sub-vital state. Chromatin stabilizers such as Mg^{2+} (Galbraith et al. 1983) or spermine (Bino's buffer, Doležel's LB01 buffer) are added. Mannitol and sucrose are used to provide isotony. Chelators such as EDTA bind metal ions and thus block DNase activity (DNases need Mg^{2+} and Mn^{2+}). Citrate acts as a chelator as well. Thus, Mg salts as stabilizers combined with chelators as DNase inhibitors seems to make little sense. Some buffers contain mercaptoethanol, sulphite, ascorbic acid and dithiothreitol as reductants, and PVP to bind tannins (see below).

The different buffer characteristics and the cytosolic compounds released upon chopping up the tissue can affect sample and measurement quality. Comparative analyses of buffers are therefore required, but such studies have seldom been undertaken.

Recently, Loureiro et al. (2006a) compared four common and chemically different lysis buffers, namely Galbraith's buffer (Galbraith et al. 1983), LB01 (Doležel et al. 1989), Otto's buffer (Doležel and Göhde 1995) and Tris.$MgCl_2$ (Pfosser et al. 1995), taking into consideration the following parameters: fluorescence yield of nuclei in suspension, CVs of G_1 peaks, forward and side scatter, amount of debris, and the number of particles released from the sample tissue. Samples were prepared from fresh leaf tissue of seven plant species covering a wide range of genome sizes (1.30–26.90 pg/2C), differing in tissue structure and being either easy to prepare (*Pisum sativum*, *Vicia faba* and *Lycopersicon esculentum*) or more challenging (*Oxalis pes-caprae*, Oxalidaceae, complicated by acidic cell sap; *Celtis australis*, Ulmaceae, complicated by mucilage, *Festuca rothmaleri*, Poaceae, complicated by xeromorphic, and *Sedum burrito*, Crassulaceae, complicated by succulent leaves).

The buffers performed differently, although with acceptable results in most cases. Excellent results (high fluorescence yield, high nuclei yield, low CV, little debris) were obtained only with some buffers for some species. *Oxalis pes-caprae* with very acidic cell sap worked only with Otto's and Galbraith's buffer. Spermine (in LB01) seems to be a better chromatin stabilizer than $MgSO_4$, and MOPS (in Galbraith's buffer) seems to be a better buffer substance than Tris (evident in the acidic *O. pes-caprae*). A higher concentration of detergent (0.5% Triton X-100) was essential for the improved performance of Tris.$MgCl_2$ buffer in *Celtis australis* which contains a high level of mucilage. Generally, the results obtained with Otto's buffer were excellent (nuclei had high relative fluorescence intensity and the lowest CV values) in many species. An exception was the grass *Festuca roth-*

maleri, a technically difficult taxon to work with, which produces less satisfactory results with Otto's buffer and Tris.$MgCl_2$. Loureiro et al. (2006a) even found that for a given species the analysis of scatter properties (FS and SS) of nuclei provides a ''fingerprint'' of each buffer.

The finding that LB01 buffer, which contains Tris as the buffer substance, performed very well while Tris.$MgCl_2$ buffer yielded the least satisfactory results (with exceptions), shows that it is probably not the buffer substance itself which makes a good isolation buffer, but its concentration and the additives such as chromatin stabilizers and antioxidants, ionic strength, and detergent concentration.

Which buffer is preferable? Loureiro et al. (2006a) showed that of the four lysis buffers used, none gave consistently good results with all seven species tested. Although LB01 and Otto's buffer are recommended as the first choice, it is worthwhile testing various buffers to identify the best one for a given material. Notably, Loureiro et al. (2006a) also documented some slight differences in relative fluorescence yield depending on which buffer was used. This would mean that it may be the buffer which causes some divergence between laboratories in the estimation of genome size of the same material. The reasons for this divergence are therefore unclear and deserve investigation.

4.5 Standardization

It is self-evident and long known in DNA cytometry, that data can seldom be used straight from the machine (Bennett and Smith 1976). To make data widely comparable and thus useful, there must be some reference, that is, a biological sample having known parameters of interest, with which the unknown sample is compared. This reference material is known as the *standard*. The standard may already be present endogenously, such as in cases where in the same test material a certain type of nuclei functions as the reference for other nuclei (*endogenous standard*, for example in endopolyploidy studies). Otherwise, the standard must be added. Standardization can be performed at different levels of stringency and with different aims.

4.5.1 Types of Standardization

There are different meanings attached to the word ''standard''. Often a set of rules for executing a method or preparing a reagent is called the standard, but in our context standard mostly means biological material included in the procedure to compensate for the technical variables and imponderables as far as possible, so that the true relationship between the unknown and the standard is revealed and universal comparability is (hopefully) achieved. Fluorescent beads are an example

of an abiotic or physical reference for instrument setting and are included in tests to calibrate the instrument gain or to serve as a staining-insensitive landmark in histograms.

The *biological standard* is a biological material with similar characteristics to those of the unknown sample, which can be measured in the same way, so that comparison and conversion of data is possible and a reference material is appointed for forthcoming experiments. Application can be as external or internal.

The *external biological standard* is not included in the sample to be measured, but the conditions of sample preparation are kept as similar as possible for both the unknown sample and the standard. In *Glycine max* (Fabaceae) DNA content studies have been undertaken in which the instrument was calibrated in the morning for a certain peak position of the external standard (a soybean cultivar), and for the rest of the day a number of cultivars were measured at constant machine settings. It was assumed that variation in peak position up to 1.12-fold indicated differences in DNA content, as opposed to technical fluctuations (Graham et al. 1994; Rayburn et al. 1997). It is clear that such an assumption would have been more justified had the standard always been co-processed with the sample (cf. Table 4.4). Other authors using the latter approach could not confirm this variation (Greilhuber and Obermayer 1997; Obermayer and Greilhuber 1999). External standardization is acceptable when the demands of precision are not high, as in DNA ploidy screening.

The *internal biological standard* is included in the same experiment to guarantee as far as possible identical conditions for the unknown sample and the standard during the whole procedure of preparation, staining and evaluation. Here, of particular relevance are the secondary metabolites of plants (often phenolics) which bind to chromatin. Acting as a steric barrier for fluorochrome binding they modify peak shape and position (Price et al. 2000; the Report on the IBC Workshop on Genome Size in Bennett and Leitch 2005; Loureiro et al. 2006b). If the secondary metabolites do act in these ways, then they ought to influence both the unknown and the standard, in as similar a manner as possible. Consequently, if the standard and sample are chopped up together then the standard should be inhibited by the secondary metabolites to a similar degree as the sample, so that the calibrated value of the unknown is more or less rectified (with emphasis on *more or less*). This is also the basis of a test for inhibitors (see below).

Some authors have used a type of standardization that is intermediate between external and internal standardization, i.e. isolating standard and sample independently and mixing the isolates, or adding the standard to the stained sample isolate after having cleaned the stained sample by centrifugation and replacing the old dye with a fresh one (Johnston et al. 1999). The standard is then stained in an environment free from the inhibitors present in solution, but which have already influenced the sample nuclei during staining. Not surprisingly, its peak quality may be better, but the relationship to the unknown sample peak is not any more authentic. Such a procedure may be termed *pseudo-internal* standardization (Noirot et al. 2005) and is approaching external standardization.

Table 4.4 Covariation of DNA content values upon internal standardization in *Secale cereale* ''Elect'' (the unknown) and *Pisum sativum* ''Kleine Rheinländerin'' (the standard) (Otto procedure, propidium iodide staining at 50 mg l^{-1} overnight). One co-isolate was divided into two aliquots (tubes a and b) and measured in steps as indicated. AU, 2C peak position at gain 551 on the Partec PAII. Conversion to pg was based on 1C = 4.38 pg for *P. sativum*. For details see text.

Time (min)	*P. sativum* 2C, AU	*S. cereale* 2C, AU	Ratio	*S. cereale* 1C, pg
0	54.65[a]	98.96[a]	1.811	7.931
7	55.06[a]	99.75[a]	1.812	7.935
15	55.69[a]	100.83[a]	1.811	7.930
24	56.52[a]	102.00[a]	1.805	7.904
37	56.86[a]	103.85[a]	1.826	8.000
42	52.17[b]	94.84[b]	1.818	7.962
46	52.27[b]	94.86[b]	1.815	7.949
52	52.83[b]	95.93[b]	1.816	7.953
57	49.95[b]	90.48[b]	1.811	7.934
63	50.63[b]	91.65[b]	1.810	7.929
Mean	53.66	97.32	1.813	7.943
SD			0.006[c]	0.026[c]
CV (%)			0.321[c]	0.321[c]
SD	2.43	4.45	0.117[d]	0.512[d]
CV (%)	4.53	4.57	6.435[d]	6.435[d]

[a] test tube a.
[b] test tube b.
[c] SD and CV based on co-chopped ratios.
[d] SD and CV based on ratio of species sums.

4.5.2 Requirement of Internal Standardization – a Practical Test

The importance of internal standardization is highlighted by the test shown in Table 4.4, in which *Secale cereale* ''Elect'' (Poaceae), the ''unknown sample'', is compared with *Pisum sativum* ''Kleine Rheinländerin'', the standard. One co-chopped isolate was divided in two parts (tubes) and processed. Each tube was measured five times in sequence. While the absolute variation in arbitrary units was up to 1.138-fold in pea and 1.148-fold in rye, variation of the rye/pea ratio reached a maximum of 1.012-fold, at a coefficient of variation of 0.3% between runs. The resulting rye/pea ratio of 1.813 differs only slightly from the average 1.779-fold found in *S. cereale* ''Dankovske'' by four laboratories in a ring-study on plant standards (Doležel et al. 1998) and coincides with the 1.813-fold found by laboratory 3 in the quoted study (with a different operator and using a differ-

ent type of lamp-based instrument). Had single absolute values been used in an arbitrary manner, up to 1.307-fold variation could have been stated for the unknown.

It should be noted that the non-standardized variation within pea and rye reported here is in the range of the ''intraspecific variation'' between cultivars described in studies where the authors did not use internal standardization (Graham et al. 1994; Rayburn et al. 1997). Therefore, internal standardization is a necessity even when no fluorescence inhibitors are present. There are variables in the procedure of isolation, staining and measurement, which without internal standard could be controlled only with difficulty. Such variables include temperature and time of staining, dye concentration, pH shifts due to cell sap, and quantity of material.

4.5.3 Choice of the Appropriate Standard Species

Standard species should fulfil several criteria.

4.5.3.1 Biological Similarity

The researcher should be able to prepare the standard material synchronously together with the unknown sample, and the materials should be biologically similar. Fixed chicken red blood cells (CRBCs), human leucocytes or salmon sperm can thus hardly be regarded as an ideal internal standard for determination of genome size in plants. CRBCs are commercially available or are self-prepared, fixed and stored, often for years at low temperatures. Such material then often has a history different from the plant samples to be tested. There are no targeted studies known which could have proven the full reliability of this type of material, but there are indications that caution is appropriate. Johnston et al. (1999) report 2C = 2.49 pg for CRBCs kept at Texas A&M University, and 3.02 pg for chicken cells kept at Arizona University (a beetle *Tetraodes* sp., Caraboidea, with 2C = 1.0 pg was the standard). This is a 1.21-fold variation which seems to have been reproducible in their study. The genome of a male chicken (with ZZ constitution) is 2.7% larger than that of a female (with ZW constitution) (Tiersch et al. 1989). Galbraith et al. (1983) provided a more recent chemical determination of the DNA content of CRBCs and arrived at $2C = 2.33 \pm 0.22$ pg (mean $\pm$ SD, $N = 7$), meaning a 95% confidence interval between 2.167–2.493 pg. Bennett et al. (2003) co-ran chicken and *Arabidopsis thaliana* and estimated about 15% less DNA in the 2C peak of the bird than in the 16C peak of the plant (2.569 pg), indicating 2C = 2.233 pg for chicken. This value is lower than commonly accepted values between 2.33 and 2.5 pg (cf. Bennett et al. 2003), but is within the 95% confidence interval of the chemically-determined value given by Galbraith et al. (1983). Based on the data by Tiersch et al. (1989), male human leucocytes should have 2C = 6.278 pg, because the chicken/human ratio is 0.3557. The sex of the two chicken samples (2C = 2.45 and 2.53 pg) was not given by Tiersch et al. (1989), but their mean values are used here.

The data of Bennett et al. (2003) indicate a CRBC/*Arabidopsis* ratio of 6.960, whereas the data of Ozkan et al. (2006) indicate a value of 5.224. Whilst Bennett et al. (2003) compared the genome size of these organisms and co-prepared their material, Ozkan et al. (2006) primarily compared the genome size of di- and tetraploid *A. thaliana* lines and used CRBCs as a reference for staining intensity without explicitly mentioning co-preparation. This 1.33-fold discrepancy may be at least partly caused by a staining artifact of the CRBCs.

4.5.3.2 **Genome Size**

The standard species should be different in genome size from the unknown sample, but not too different to avoid instrumental problems with linearity. The peaks of the standard should not overlap with the peaks of the unknown sample. NB at high N, say 1500, and normal distribution, the range of a sample can be estimated by SD × 6, where 99.7% of the values are included (Sachs 1978, p. 79). The difference between the standard and the sample should thus be equal to or exceed the threefold sum of both standard deviations. The minimum difference should be about 20% when the CV is about 3%. Linearity problems with FCM are the main reason why a single DNA standard species in plants cannot be sufficient for the nearly 2000-fold range in C-values.

4.5.3.3 **Nature of the Standard**

Ideally the standard species should be free of fluorescence inhibitors, and its preparation should be unproblematic so that its analysis should result in narrow peaks. Thus, colored or mucous-containing plants or plant organs appear *a priori* to be inappropriate. Infected plants should be rejected, because they may be stimulated to produce inhibitors. A procedure for checking for inhibitors is given below.

4.5.3.4 **Availability**

Permanent availability of seed or plant material should be guaranteed for continuous experimental work. Seeds should germinate easily. Opinions differ with regard to the strictness which should be applied to selecting standards. Some authors favor a few elite standards (i.e. selected breeds of a few species; see below). For instance, M. D. Bennett et al. (personal communication) recommend for the future a mutant of *Arabidopsis thaliana* which has no flavonoids (inhibitors), and whose endopolyploid nuclei can be used as reference points in addition to the 2C and 4C peaks (cf. Chapter 7). Other authors assume a more pragmatic standpoint. We believe that laboratories which have no resources for breeding standard species themselves can obtain suitable material from reliable distributors. This material can then be calibrated with elite standards. For example, a variety of vegetable pea common in a country (e.g. *Pisum sativum* "Kleine Rheinländerin" in Austria) can be calibrated with *P. sativum* "Minerva Maple", a standard used and recommended by Bennett and Smith (1976), or with *P. sativum* "Ctirad", as suggested by Doležel et al. (1998). But note that *P. sativum* "Minerva Maple" is a field pea with colored flowers and possibly higher phenolic content than vegetable

peas. Greilhuber and Ebert (1994), Baranyi and Greilhuber (1995, 1996) and Baranyi et al. (1996) have shown that the genome size of *P. sativum* is stable worldwide. These authors concluded this from the fact that land races and even wild accessions from extremely different climates did not differ in C-value from highbred cultivars. Why should authors be restricted to a certain pea line of limited availability, when probably any vegetable pea (i.e. the white-flowering variety) will fulfil the same criteria? Likewise, the genome size of *Glycine max* is apparently universally stable (Greilhuber and Obermayer 1997, 1998a; Obermayer and Greilhuber 1999). Recent reports of some marginal variation between lines (Chung et al. 1998; Rayburn et al. 2004) should be reconsidered in the light of the effect of fluorescence inhibitors. In the case of Rayburn et al. (2004), the low variation found (ca. 3%) may rather depend on the anthocyans present in the hypocotyls used for the measurements and in addition the results were not confirmed using rigorous statistical testing (only the LSD test was applied). Chromosomally engineered and hybrid strains of modern cereal varieties, and also onions, should be used cautiously. It is more meaningful to use old-established lines.

4.5.3.5 **Cytological Homogeneity**

The standard and sample should be cytologically fairly homogeneous. Seedlings from aged seeds can be problematic because of mitotic aberrations.

4.5.3.6 **Accessibility**

Standards used should be accessible to other researchers, that is, should be distributed upon request in sufficient quantity.

4.5.3.7 **Reliability of C-Values**

A reliable C-value should be established, optimally based on measurements by different laboratories. This is a sensitive point, because in fact only one C-value for a plant standard evaluated using a method yielding absolute amounts of DNA is generally accepted. This is *Allium cepa* (Alliaceae), whose nuclear DNA content per root tip meristem cell (expectedly corresponding to roughly 3C) has been chemically determined as 54.3 pg by Sparrow and Miksche (1961) and was re-calculated as 2C = 33.55 pg by Van't Hof (1965) who took into account the relative lengths of the mitotic cycle phases. This value agrees well with chemically determined values obtained from animals and humans using the Feulgen cytophotometric comparison (Greilhuber et al. 1983). Almost all other trustworthy C-values for plants are based on cytometric comparisons with plants and lastly with onion, or with human and animals, for which chemical estimates exist. The old chemical estimates in the human vary between 1C = 3.0 and 3.5 pg (Métais et al. 1951; Vendrely and Vendrely 1949). Many authors arbitrarily used the higher value for their calibrations, although a value of 3.1–3.2 pg may be closer to the truth (Doležel et al. 2003; Greilhuber et al. 1983). In one important recent investigation (Bennett et al. 2003), the size of a completely sequenced genome size was already known, that is, of the nematode *Caenorhabditis elegans*, which was used for FCM

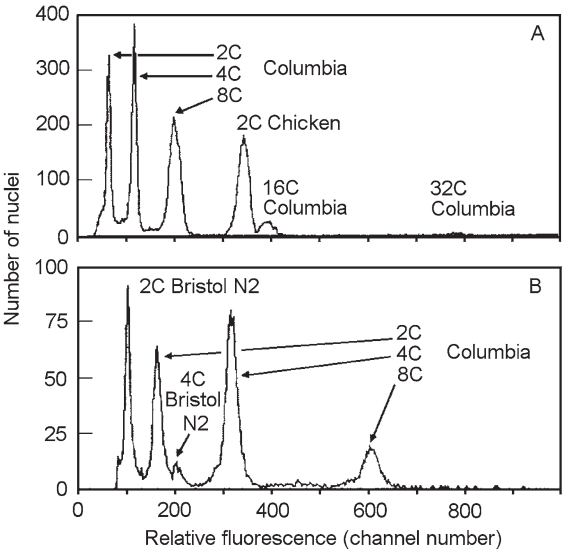

Fig. 4.3 Simultaneously prepared and measured propidium-iodide stained nuclear suspensions of *Arabidopsis thaliana* "Columbia" and chicken (a) and *Caenorhabditis elegans* "Bristol N2" (b), respectively. The positions of chicken 2C relative to *A. thaliana* 16C and of *C. elegans* 4C versus *A. thaliana* 2C give an indication of the genome size of *A. thaliana* and chicken on the basis of a *C. elegans* 1C-value of 100 Mbp. For details see text. (From Bennett et al. 2003 with permission).

comparison with *Arabidopsis thaliana* "Columbia". For this important plant species, a value of 1C = 157 Mbp was estimated using FCM, based on 1C = 100 Mbp for this worm (Fig. 4.3). This example clearly showed the fragility of the value of 125 Mbp published by the Arabidopsis Genome Initiative (2000), which significantly underestimated the non-sequenced DNA harbored in the heterochromatin (Bennett et al. 2003). But note that *A. thaliana* collected in the wild was meanwhile reported to vary by about 10% in genome size between accessions (Schmuths et al. 2004). There is clearly a need for in-depth analyses of genome sizes of plant standard species to arrive at agreed absolute values.

4.5.4 Studies on Plant Standards

Doležel et al. (1998) were the first to compare a set of nine different standard species of defined cultivars or lines in four laboratories with PI and also with DAPI, and laser and lamp-based flow cytometers, and with Feulgen scanning densitometry. The species were compared in a cascade-like manner starting from *Allium cepa* (assumed to be 2C = 33.55 pg) down to *Arabidopsis thaliana*, with a mean result of 2C = 0.37 pg, while 0.321 pg is the expected value reported by Bennett

Table 4.5 Ratios of C-values and relative standard deviations ($N = 10$) estimated for pairs of species by four laboratories (L1–L4). Nuclei were isolated simultaneously and stained with propidium iodide. A.c. *Allium cepa*, V.f. *Vicia faba*, S.c. *Secale cereale*, H.v. *Hordeum vulgare*, P.s. *Pisum sativum*, Z.m. *Zea mays*, G.m. *Glycine max*, R.s. *Raphanus sativus*, A.t. *Arabidopsis thaliana*. (Adapted from Doležel et al. 1998).

	Ratio of C-values (CV%)							
	V.f./ A.c.	**S.c./ V.f.**	**H.v./ S.c.**	**P.s./ H.v.**	**Z.m./ P.s.**	**G.m./ Z.m.**	**R.s./ G.m.**	**A.t./ R.s.**
L1[a]	0.778 (0.9)	0.613 (1.0)	0.647 (0.6)	0.874 (1.0)	0.639 (3.3)	0.469 (6.6)	0.506 (1.2)	0.310 (1.0)
L4[a]	0.792 (3.5)	0.606 (2.8)	0.661 (0.8)	0.869 (0.9)	0.658 (2.9)	0.519 (0.8)	0.464 (0.6)	0.302 (0.3)
L2[b]	0.776 (1.3)	0.595 (0.8)	0.638 (0.8)	0.863 (0.8)	0.609 (1.3)	0.441 (1.6)	0.462 (1.7)	0.300 (0.7)
L3[b]	0.752 (2.1)	0.586 (1.4)	0.632 (0.8)	0.879 (0.5)	0.586 (0.5)	0.438 (0.8)	0.465 (1.9)	0.313 (3.5)
Mean ratio	0.774	0.600	0.645	0.870	0.623	0.467	0.474	0.306
Largest difference between laser cytometers (%)	1.8	1.1	2.1	0.6	2.9	9.6	8.3	2.6
Largest difference between lamp cytometers (%)	3.1	1.5	0.9	1.8	3.8	0.7	0.6	4.2
Largest difference (all instruments) (%)	5.1	4.4	4.4	1.8	10.9	15.6	8.7	4.2

[a] Laser-based instruments.
[b] Lamp-based instruments.

et al. (2003). Feulgen DNA measurements with 2C = 0.326 pg closely approached this value. The four laboratories produced strongly correlated data although the types of cytometer used differed in that laser instruments seemed to slightly underestimate the larger genomes. Nevertheless, some critical differences between laboratories were noticed (Table 4.5). Ratios of single species pairs differed by up to 15.9% (mean 6.9%), which was higher than anticipated. Laser instruments produced results which differed by up to 9.6% (mean 3.6%), and with lamp-based instruments the results differed by up to 3.8% (mean 2.1%; Table 4.5). These differences are difficult to explain but may be related to instrument-specific linearity bias, differences in the growth conditions of the plants, the use of different plant parts and perhaps also to the use of different buffers, which according to Loureiro

et al. (2006a) can influence the various species investigated somewhat differently (see above).

Johnston et al. (1999) conducted a study on plant standards for FCM involving two laboratories, using among other crop species *Pisum sativum* ''Minerva Maple'', *Hordeum vulgare* ''Sultan'' (Poaceae), *Vicia faba* ''GS011'', and *Allium cepa* ''Ailsa Craig''. This study revealed problems with CRBC variability, and compared with Doležel et al. (1998) generally yielded somewhat higher 2C-values for *P. sativum* (9.56 vs. 8.75 pg), *H. vulgare* (11.12 vs. 10.04 pg), and *V. faba* (26.66 vs. 25.95 pg). The value for *Allium cepa* was accepted to be 33.55 pg. As already mentioned, in this study, the beetle *Tetraodes* sp. (2C = 1.0 pg) was the primary standard; it served for two chicken accessions whose 2C-values were quite different i.e. 2.49 and 3.01 pg. Of these, the higher (and probably too high) value of 3.01 pg was used for calibrating *H. vulgare*, which was then used to calibrate the remaining species (Johnston et al. 1999). It seems that assuming a too high value for the chicken is the main reason for the higher plant DNA values given by Johnston et al. (1999) compared to Doležel et al. (1998).

4.5.5 Suggested Standards

A widely used standard is *Pisum sativum*, but the absolute values which have been assigned to it are divergent; this is in sharp contrast to the findings of Baranyi and Greilhuber (1995, 1996) that the genome size of *P. sativum* is stable worldwide. *Pisum sativum* has the advantage of being intermediate in genome size among angiosperms, poor in or devoid of inhibitors, well established for genome size stability, and neither rich in nor completely devoid of heterochromatin. It is easily available and germinates fast, and responds equally well to different isolation buffers (Loureiro et al. 2006a). Therefore, it has all the qualifications of a primary standard, against which secondary standard species can be calibrated. Its 1C-value is presently best taken as 4.38 pg or 4.284 Gbp, which is the mean value obtained by four laboratories using laser and lamp-based flow cytometers (Doležel et al. 1998). A very similar 1C-value of 4.42 pg has been measured with Feulgen densitometry by comparison with *Allium cepa* (Greilhuber and Ebert 1994). Marie and Brown (1993) report an almost 5% lower value (i.e. 4.185 pg/1C) for *P. sativum* ''Express Long'', when calibrated with *Petunia hybrida* ''PxPc6'' (1.425 pg/1C, Solanaceae), which had been calibrated with female CRBCs (1.165 pg/1C). Doležel et al. (1998) assumed 1C = 4.545 pg for *Pisum sativum* after calibration against human leucocytes with 1C = 3.5 pg, but the latter value seems to be the upper limit for the human (see above).

Thus, there is great interest in a unique standard which fulfils all demands – the ''plant gold standard'', against which all other plant standards can be calibrated. An *Arabidopsis thaliana* mutant with knocked-out flavonoid production is being reviewed as a potential standard (M. D. Bennett et al., personal communication), in which the 2C, 4C, 8C, and 16C peaks could be used, the first peak representing 0.321 pg DNA (314 Mbp), the final peak, 2.569 pg (2.512 Mbp).

However, reduced peak height at the higher C-levels may limit the use of this species as a standard. While such a ladder meets the most frequent 2C-values in angiosperms, higher C-values need other standards. It seems, that a set of standard species covering the whole range of DNA content in angiosperms cannot be circumvented. Unfortunately, consensus on a unified set of standard species with agreed C-values has not been achieved.

An overview of species used in the literature for standardization is presented in Chapter 18 (Table 18.2), and occasionally large variations of assumed C-values are recognized. A list of nine species and the values obtained by four laboratories are presented by Doležel et al. (1998). These data also give the impression of some variation between teams, notwithstanding the application of best practice rules.

4.6 Fluorescence Inhibitors and Coatings of Debris

Although the interference of secondary metabolites with staining procedures had been recognized for some time in cytophotometry (Greilhuber 1986), it was not until Noirot et al. (2000) and Price et al. (2000) published their findings that this effect was taken seriously in plant FCM. Until recently, this interference was thought to be fluorescence inhibition, but research carried out in the meantime appears to suggest that there are additional effects such as the aggregation of minor particles with nuclei that also play a role in this interference and can even lead to an apparent increase in nuclear fluorescence (Loureiro et al. 2006a). The role of autofluorescing metabolites is still hypothetical and needs investigation. Therefore, we distinguish here between *inhibitors* and *coatings of debris*, the latter being particles of endogenous substances sticking to the nuclei, resulting in a deterioration of the quality of the FCM histogram peaks without necessarily decreasing the overall nuclear fluorescence.

4.6.1 What are Fluorescence Inhibitors and Coatings of Debris?

The chemical identities of fluorescence inhibitors are poorly explored, but in many instances phenolic substances possessing active hydroxyl groups (providing free electrons capable of forming hydrogen bonds) are most probably involved. Such compounds can consist of glycosylated or non-glycosylated monomers (e.g. anthocyans, flavonoids), oligomers, and polymers. Condensable tannins and the hydrolyzable tannins (mainly gallotannins and ellagitannins) are the more widely known types of the polymers. In the reduced state, these phenolics often show little or no color, and they form strong hydrogen bonds with carboxyl groups of proteins and probably also with DNA (Walle et al. 2003). Polyhydroxyphenols (phenolics with two or more active hydroxyl groups) can crosslink proteins. Tan-

nins (for tanning leather) are large polymeric molecules which are able to cross-link the collagen fibres of skin (Endres 1961); these bonds can be disrupted with 8 M urea. Also heat, high pH and the compound Dioxan (ethylendioxid, $C_4H_8O_2$) can act as tannin strippers. When hydroxyphenols are oxidized, a quinone structure is formed which often results in browning or coloring of the compound. Such quinones are highly reactive species themselves and form covalent bonds with carboxyl groups (Endres 1961). Such bonds are irreversible, while hydrogen bonds are reversible. When nuclear suspensions turn brown or show precipitation, the presence of phenolics is evident. Workers have added antioxidants such as β-mercaptoethanol (a component of Doležel's LB01 buffer, see Table 4.3; cf. also Chapter 18), ascorbic acid or sodium metabisulphite to the isolation buffer to keep any phenolics (which are reductants themselves, that is, are easily oxidized) in their reduced state (e.g. Bharathan et al. 1994). Any hydrogen bonds could then hopefully be maintained in their reversible state and disrupted by the addition of a competitor. An example of such competitors is the low-molecular weight polyvinylpyrrolidones (PVPs); for reasons of viscosity the lower molecular weight classes (e.g. PVP-10, PVP-40) are used in FCM. Note that the monomer, vinylpyrrolidone, is highly hazardous, while the polymer is harmless. PVPs are not reductants but their amide groups are available for binding with inhibitors, in competition with those of the proteins and DNA (Gustavson 1963). PVPs are used in biochemistry, whenever problems caused by secondary plant metabolites occur, especially in protein electrophoresis and in DNA extraction procedures (e.g. Friar 2005). PVPs can reactivate enzymes which have been inactivated by phenolics (Schneider and Hallier 1970) and are widely used in beverage production as an absorbent for tannins. It seems reasonable to combine a PVP with antioxidants in nuclear isolation buffers to allow the phenolics to be stripped from proteins and DNA *before* they become oxidized. Once oxidized, phenolics, as quinones, bind covalently and practically irreversibly to the carboxyl groups, a situation which should be prevented. Bharathan et al. (1994) observed positive effects of PVP on histogram quality. Yokoya et al. (2000) found that a minimum of 10 g l^{-1} of PVP-40 greatly improved the quality and fluorescence intensity of DAPI-stained co-processed preparations of parsley, as the standard, and roses, while parsley alone was unaffected. This was attributed to the phenolics in the leaves of roses, which also influenced the standard to the same degree but in that case were absorbed by the PVP.

The effect of cytosol on PI fluorescence in *Coffea* (Rubiaceae) was demonstrated by Noirot et al. (2000, 2002, 2003, 2005). Cytosol from *Coffea* leaves and defined components such as the phenolic chlorogenic acid, reduced the fluorescence yield of *Petunia hybrida* nuclei which was used as the non-phenolic standard. Elevating the temperature of nuclear isolates before staining changed the relative fluorescence values of *Coffea* and *Petunia* by decompaction of chromatin which enhances fluorochrome binding. Addition of caffeine was able to partly restore the fluorescence yield of quenched *Petunia* nuclei (Noirot et al. 2003), which may be explained by the known gallotannin-binding property of caffeine.

That phenolics bind to DNA is clearly evident from results with purified DNA. A binding mechanism for phenolic monomers has been proposed by Sarma and Sharma (1999), who observed the direct complexation of cyanidin with calf thymus DNA, suggesting that it was the positively-charged cyanidin molecule which associates with the negatively-charged phosphate groups of the DNA backbone. Walle et al. (2003) investigated the binding of quercetin to protein and DNA using human intestinal and hepatic cells as the targets, and demonstrated the covalent binding of quercetin to the DNA following peroxidase-induced oxidation. The covalent binding of quercetin to protein (75–125 pmol mg^{-1}) was stronger than that to DNA (5–15 pmol mg^{-1}).

Ellagic acid is a highly efficient DNA-binding polyhydroxyphenol and belongs to a class of hydrolyzable tannins known as ellagitannins. It is abundant in certain fruits, for example, in strawberries and raspberries, and has anticancer activity, which can be explained by its anti-methylation properties resulting from a double-helical DNA affinity binding mechanism, rather than by an oxidant-scavenging mechanism (Dixit and Gold 1986).

Whitley et al. (2003) administered ^{14}C-labeled ellagic acid to cultured intestinal human cells and found a rapid, intense and irreversible binding to macromolecules. Proteins were crosslinked (which was not found to the same extent with quercetin; Walle et al. 2003), whereby irreversible binding required oxidation of ellagic acid. However, five times more ellagic acid was bound by DNA (5020 pmol mg^{-1} DNA) than by proteins (982 pmol mg^{-1} protein). This binding to DNA was irreversible but did not require oxidation of ellagic acid. Ellagic acid seems to be firmly bound to DNA by an intercalation mechanism (Whitley et al. 2003). From the foregoing it appears that ellagic acid could be a major factor in nuclear fluorescence quenching as observed with FCM.

Another class of phenolic compounds of concern are the coumarins, which intercalate into DNA and cause ApT adducts and crosslinks after UV irradiation (Sastry et al. 1992). Walker et al. (2006) associated variable DNA values in *Bituminaria bituminosa* (Fabaceae) with temperature-dependent variation of furanocoumarins in this species.

There are reports that phenolics, such as flavonoids and flavanols, are present *in vivo* within plant nuclei (Feucht et al. 2004). It appears probable that the finding of conspicuous flavanol content (evidenced by dark-blue coloring) of plant nuclei (of trees such as conifers, *Coffea* and *Prunus*) after *in vivo* application of the DMACA reagent (i.e. 1 g 4-dimethylaminocinnamaldehyde dissolved in 100 ml 1.5 N sulfuric acid) is an artifact, although the authors put forward arguments for *in vivo* binding (Feucht et al. 2004; and the preceding literature). While cells die and cell membranes break down, especially under acidic conditions, vacuole-located condensable tannins penetrate all surrounding tissue and are attached conspicuously to nuclei and chromosomes. Note that at the same time tannins act as a strong fixative, that is, the nuclei retain their shape. This is what also occurs in such plants during fixation with acidic-alcoholic fixatives or during hydrolysis of unfixed cells in hydrochloric acid (Greilhuber 1986). Clearly, *in vivo* bind-

ing of phenolics to nuclear chromatin can only be proven by analysis of living cells.

However, there is evidence in *Arabidopsis thaliana* that flavonoids are located in purportedly living cells not only in the cytoplasm, but also within nuclei. Flavonoids were stained with the fluorescent reagent diphenylboric acid 2-aminoethylester (DPBA) and appeared in nuclei in plasmolyzed cells in the root elongation zone (Peer et al. 2001). Plasmolysis was obviously elicited to test the vital status of the cells but it is not clear if the cells were alive at the time the photographs were taken. Saslowsky et al. (2005) showed flavonoid localization with DPBA in all protoplasm including nuclei of root cells, but did not mention viability. The reality of phenolic *in vivo* binding to nuclei is of importance for plant FCM and needs to be corroborated on a broad scale.

4.6.2 Experiments with Tannic Acid

Tannic acid is the glycoside of gallic acid and a common water-soluble hydrolyzable tannin or gallotannin, which is useful in heuristic experiments to investigate staining interference in FCM. Loureiro et al. (2006a) applied tannic acid in 13 concentrations (0.25–3.5 mg ml^{-1}) to nuclear suspensions of *Pisum sativum* and *Zea mays* prepared with four buffers, and checked the preparations with epifluorescence microscopy. Side and forward scatter properties were cytometrically monitored in addition to PI fluorescence. With increasing tannic acid concentration, nuclei to which debris of low fluorescence was attached could be visualized. This caused an increase in fluorescence and side scatter. A population of clumps of debris then appeared in the absence of any nuclei; the clumps of debris fluoresced more weakly than the nuclei and were of higher optical complexity. Finally, the highest tannin concentrations provoked a general precipitation of the sample. The buffers exhibited some differences in performance with tannic acid, and it is likely that this was due to higher concentration or greater efficiency of the detergent. Figure 4.4 shows examples of the so-called tannic acid effect in *P. sativum*. Figure 4.5 presents FCM diagrams from pigmented young leaves of *Rumex pulcher* (Polygonaceae) plus *P. sativum* showing a comparable effect. The side scatter discloses the fraction of nuclei with attached debris as a tail. The ''poor quality'' of such peaks is largely a consequence of the characteristics of the material. For genome size measurement in such cases, modal values should be taken instead of means, or rigorous gating should be applied (Fig. 4.5c), if more suitable parts of the plant are not available. When the clean nuclei can be sorted out on the scattergram, physical purification of nuclei is unnecessary.

The studies of mechanisms of fluorescence distortion are in their infancy, but from the information available it is likely that the bound inhibitors and debris attached to nuclei can have two main effects. First, they may provide steric barriers to fluorochrome binding and thereby cause fluorescence reduction. This presumably results in a left-hand shoulder or tail, or a shift of the whole peak to the left, if all nuclei are affected. Such a tail may be confluent with non-nuclear particle

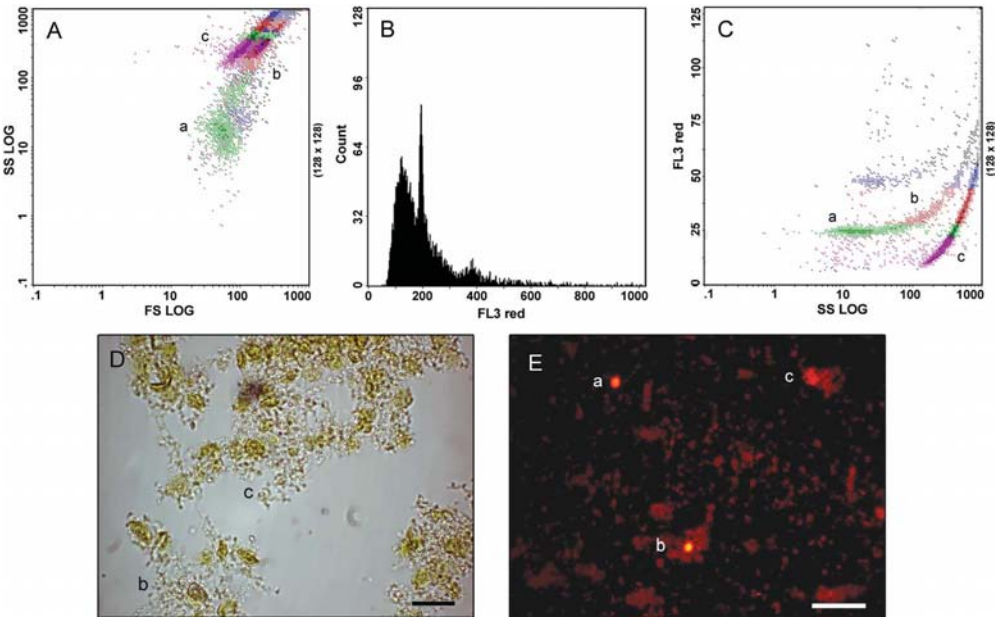

Fig. 4.4 The effect of tannic acid applied to *Pisum sativum* nuclei in suspension. Nuclei were isolated in Tris.$MgCl_2$ buffer, incubated for 15 min with 1.75 mg ml^{-1} tannic acid (TA), and stained for 5 min with propidium iodide (PI). (a) Forward scatter (logarithmic scale, FS·log) versus side scatter (logarithmic scale, SS·log) scattergram; (b) PI fluorescence intensity (FL3 red) histogram; (c) SS·log versus FL3 red scattergram; (d) bright field image after addition of TA (bar = 10 μm); (e) fluorescence image after addition of TA (bar = 20 μm, image overexposed to highlight particles with low fluorescence); a, not inhibited G_1/G_0 nuclei, b, nuclei coated with debris exhibiting enhanced fluorescence, c, fluorescent particles without nuclei. (a, c) Magenta: particles without nuclei; green, clean G_1/G_0 nuclei; brown, coated nuclei with enhanced fluorescence; blue, G_2 nuclei; gray, larger particles. (From Loureiro et al. 2006a with permission).

aggregates, as shown in Figs 4.4 and 4.5. Second, secondary metabolites may bind to nuclei and attract fluorescing debris, whereby a halo of low-fluorescing particles is created. This *coating of debris* leads to a right-hand tail or shoulder of the nuclear peaks and affects sample and standard nuclei in the same way (Figs 4.4 and 4.5). It is possible, that very large polymeric polyphenols do not penetrate the nuclei but attach externally, thus leading to more of an increase than a decrease in fluorescence. Nothing is known about other possible fluorescence quenching mechanisms, such as energy transfer.

Simple tests for the presence of phenolics are required. Such tests exist, but need to be adapted to the requirements of FCM, that is, nuclear isolates need to be tested for the presence of gallotannins, condensable tannins, ellagitannins, stilbenes, flavonoids, flavanols, coumarins, and so on. The dark-blue coloring of

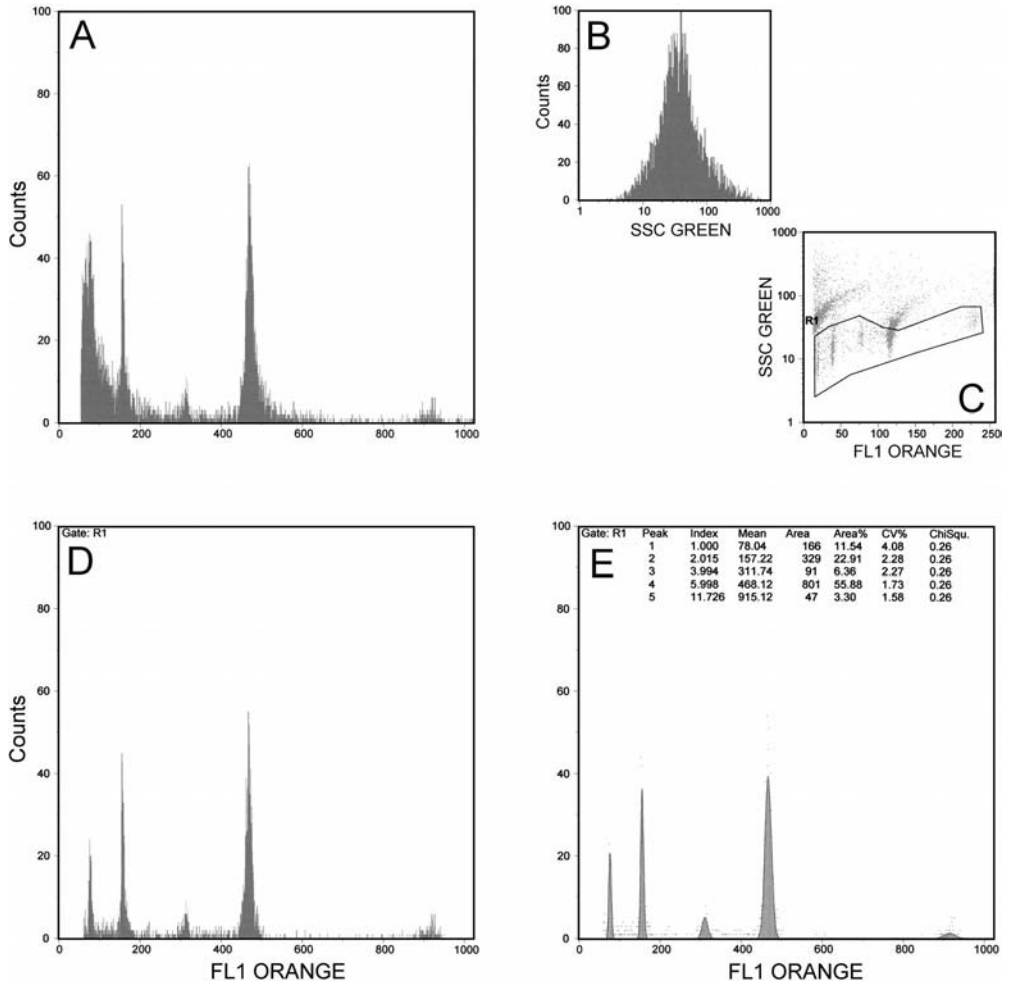

Fig. 4.5 Preparation of a very young *Rumex pulcher* leaf and *Pisum sativum* as standard, exhibiting unsatisfactory quality of the histogram and tannin-like scattergram effects (cf. Fig. 4.3). Otto's buffer, propidium iodide (PI) staining. (a) PI fluorescence histogram; (b) side scatter histogram; (c) PI fluorescence/side scatter scattergram with gating; (d) gated PI fluorescence histogram; (e) histogram with software-generated Gaussian peaks and peak parameters (peaks 1–3 belong to *R. pulcher*, peaks 4 and 5 to *P. sativum*). (Original by E. M. Temsch).

gallotannins and the green coloring of non-hydrolyzable catechin tannins obtained with ferrichloride are well known (e.g. Endres 1962). DPBA (diphenylboric acid-2-aminoethyl ester) for flavonoids (Markham 1982) and the Folin-Ciocalteu reagent for total polyphenol content (Singleton et al. 1999; Snell and Snell 1953) could also be promising reagents.

4.6.3
A Flow-cytometric Test for Inhibitors

There are examples in the literature which indicate that fluorescence inhibitors were probably involved but were at first not identified as the reason for unexpected results. Wakamiya et al. (1993) measured 19 *Pinus* species (Pinaceae) using megagametophyte and embryo tissue of *P. eldarica*. Instead of finding a 1:2-ratio between gametophyte 1C (haploid) and embryo 2C (diploid), the ratio in *P. eldarica* was 1:1.74. With Feulgen scanning densitometry the ratio was 1:1.72. However, *Pinus* embryos have tannin cells, which cause reduced staining both with Feulgen and fluorochromes, while gametophytes may have less or none. Michaelson et al. (1991), Price and Johnston (1996), and Price et al. (1998) were confronted with unprecedented DNA content variation (unorthodox genome size variation *sensu* Greilhuber 1998) in *Helianthus annuus* (sunflower; Asteraceae). At first they interpreted this variation as developmentally controlled genome downsizing and proposed the role of light quality (Price and Johnston 1996; Price et al. 1998). Later, Price et al. (2000) identified this variation as being caused by fluorescence inhibitors and described a simple test to disclose their effect. The test is based on the observation that inhibitors are released into the isolation buffer when the tissue is chopped up, and also interact with the standard nuclei. Therefore, it is necessary to compare the fluorescence intensity of the standard nuclei isolated alone with that of standard nuclei isolated together with the unknown sample. In cases where the fluorescence of the co-chopped standard appears reduced compared to the lone-chopped standard, this difference is likely to be an effect of the released inhibitor. In this way J. S. Johnston et al. (personal communication; see Bennett and Leitch 2005) elegantly demonstrated that the anthocyan, cyanidin-3-rutinoside acted as a fluorescence inhibitor in *Poinsettia* (Euphorbiaceae), in which this compound is present in red bracts but absent in green leaves.

Clearly, upon co-chopping the unknown sample is at least as strongly inhibited as the standard, if not more so. The latter could occur through the co-localization of nucleus and inhibitor in the same cell at the moment of chopping, while the standard nuclei can only be influenced by diluted inhibitor. It is thus recommended that both materials should be chopped up in a sandwich-like fashion rather than sequentially (J. Loureiro et al., unpublished results).

4.7
Quality Control and Data Presentation

The unsatisfactory situation with much of the data that had been gathered with static cytophotometry (see Greilhuber 1998, 2005) should be a warning that similar problems with FCM data should be avoided following best practice rules (cf. Chapters 5 and 7). From the foregoing it is clear that proper standardization and observation of inhibitors and coatings of debris are paramount. The highest ac-

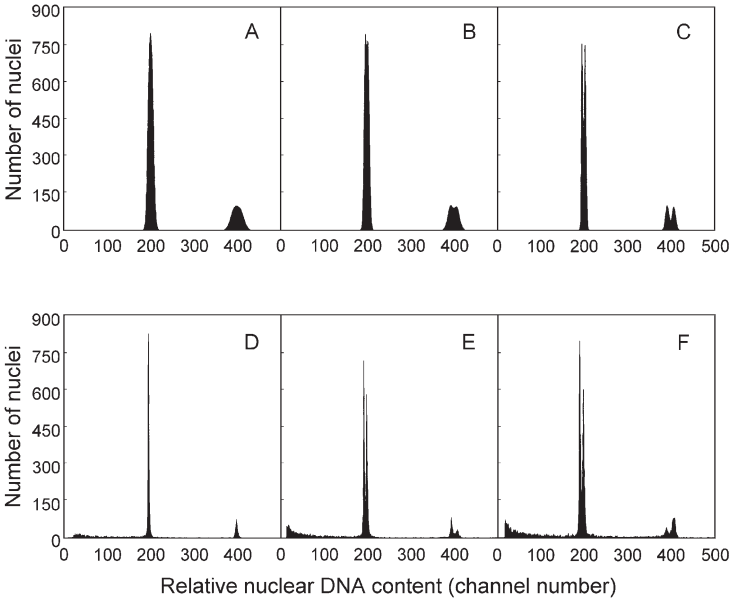

Fig. 4.6 High resolution histograms from male and female *Melandrium album* and *M. rubrum*. (a–c) Theoretical model distributions assuming a 3.7% difference between sexes at peak CVs of 3% (a), 2% (b), and 1% (c); only at CVs of 1% or lower is a clear separation obtained. (d–f) Typical histograms obtained from female *M. album* with CV = 0.53% (d), from female and male *M. album* with CV = 0.56% and 0.61%, respectively (e), and from female and male *M. rubrum* with CV = 0.70% and 0.64%, respectively (f). (From Doležel and Göhde 1995 with permission).

ceptable CV in a study, say 3 or 5%, should be set in advance. The CVs obtained should be given in some detail in the publication. Even small modern instruments measure more than one parameter. Side scatter is of much help in recognizing and eventually eliminating suspicious populations of particles. High resolution studies need stringent criteria (Suda 2004; Chapter 5). The full power of FCM has been exploited by Doležel and Göhde (1995), when the sex-difference in male and female *Melandrium album* and *M. rubrum* with XX/XY sex determination mechanism was visualized in joint preparations (Fig. 4.6). The most convincing test for true differences in DNA content is the appearance of two separate peaks in co-processed joint runs. However, this separation requires a difference of peak means of more than twice the standard deviation (Doležel and Göhde 1995).

There are rules of thumb for the required number of nuclei and the acceptable peak quality (cf. Chapter 5). Instruments are usually set to stop at 5000 to 10 000 counts, but these include G_2 and polyploid peak nuclei, perhaps S-phase nuclei, and debris depending on the sample quality and on the lower and upper level setting. Relevant peaks in general should represent ≥ 1300 nuclei. The high number of nuclei is desirable because of some fluctuation in values during a run, which

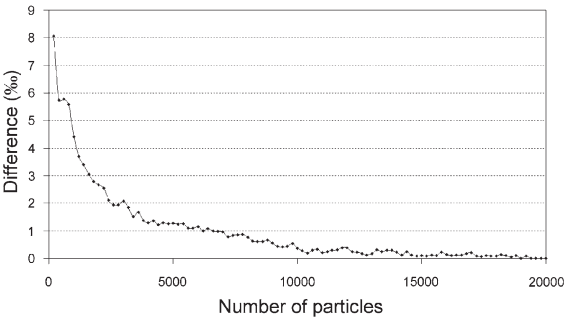

Fig. 4.7 Determination of the required number of counts to obtain stable peak position. Ten species were co-chopped and stained with DAPI (4 µg ml^{-1},10 min at room temperature) using the nearest standard species after Doležel et al. (1998). Conditions were: the species with lower genome size positioned at channel 200, 30 particles per second, about equal peak heights of standard and unknown sample, three replications per species on different days. As the measurements progressed, peak ratios were recorded at intervals of 200 counts, and after 20 000 particles the deviation from the end-value was measured. After 3000 and 7000 particles, this deviation is less than 0.2% and 0.1%, respectively. The species were: *Anthoxanthum alpinum* 2x, *Campanula patula* 4x, *Galeobdolon luteum* 2x, *Hieracium pilosella* 4x, *Oxycoccus palustris* 4x, *Pimpinella saxifraga* 4x, *Sorbus eximia* 4x, *Tragopogon pratensis* 2x, *Vicia cracca* 4x. (Courtesy of J. Suda).

should be averaged (Fig. 4.7). At a CV of 3% and 1300 nuclei the relative SE is ~0.1%. If two such peaks differ by 0.4% in position they are already statistically different ($P \leq 0.05$). Researchers should be aware that the number of nuclei per run and its CV are often not sufficiently decisive to insure the precision of a result. Independent repeats should be carried out, and the variance of these will give the measure of precision of a DNA-content determination at the level above the single preparation. The number of dependent and independent repeats should be stated in the publication. Note that statisticians regard an $N = 4$ as the lowest number of samples for meaningful statistics to be applied. Furthermore, if slightly but significantly different samples are found during an experiment, the difference should be confirmed by re-comparing these samples or accessions in independent tests. This approach has been extensively used to demonstrate the invariance of genome size in *Pisum sativum* (Baranyi and Greilhuber 1995, 1996; Baranyi et al. 1996), *Glycine max* (Greilhuber and Obermayer 1997, 1998a; Obermayer and Greilhuber 1999), and *Arachis hypogaea* (Fabaceae; Temsch and Greilhuber 2000), and to support much lower variation in *Cajanus cajan* (Fabaceae) than had been claimed previously (Greilhuber and Obermayer 1998b). Šmarda and Bureš (2006) considerably substantiated their finding of intraspecific genome size variation in *Festuca pallens* (Poaceae) by comparing results on the same accessions obtained in spring and autumn of different years and obtained with DAPI and PI, which were all highly significantly correlated.

When genome sizes are correlated with other parameters, for instance altitude above sea level of the locality of collection or mean annual precipitation, indepen-

dence of the correlated data has to be ascertained. For example, the genome sizes of populations within a species are not independent at the level of genera. A correlation of C-values with another parameter over an area of a genus with several species should be carried out using the mean values of the species, and not of the populations. The principle that forms the basis of all quality control and best practice rules is that the reader of the publication should be able to understand what has been done by the investigator.

4.8 Future Directions

There are two broad and anastomosing avenues of plant nuclear DNA content research. On the one hand we see the application of techniques available to biological questions of genome size variation such as inter- and intraspecific variation, its functional meaning, possible selective factors, directed changes in evolution, and application to systematics. These topics are covered in Chapters 5, 6, 7, 12 and 15. On the other hand we see the research in optimizing techniques, from which these biology-oriented research avenues are profiting.

Methodologically, the identification of fluorescence inhibitors and compounds causing debris-coatings, the mechanisms of their action, the degree of their effect, and finding remedies is a big challenge. Loureiro et al. (2006a) give an example of the importance of parallel light-microscopic analyses. We can conclude that plant phenolics are certainly among the secondary metabolites which constitute the main part of the problem. A set of phytochemical tests for the presence of phenolics in nuclei isolates needs to be worked out. The inhibitor test for peak shifting (Price et al. 2000) should become standard in plant FCM work. The problem of minor intraspecific DNA content variation could be much better evaluated if such tests were routinely carried out.

Another technical challenge is the utilization of conserved (dried, fixed) plant material for FCM. There are thousands and thousands of fixed cytological samples in deep freezers in botany laboratories all over the world. DNA in fixed cell nuclei stored in ethanol at low temperatures (≤ -20 °C) remains stable over many years (Greilhuber and Temsch 2001). Routine techniques for applying meaningful FCM to this kind of material seem to be realistic but are still not available.

Recent work (Suda and Trávníček 2006) has shown the feasibility of carrying out FCM on herbarium material in certain plant groups for up to 2 years at least (cf. Chapter 5). This opens up new perspectives for analyzing field-collected material. It is a common experience that for the botanist the shortage of time in the field is the major obstacle to preparing suitably fixed cytological samples for densitometric DNA content studies. FCM of herbarium or silica gel-dried material (now used routinely for DNA studies) could become a popular alternative to densitometry. Research into the reasons for the decay in the quality of dried material, and how to slow it down or to overcome it, is urgently needed.

The recent publications reporting the use of dormant seed material for FCM (Matzk et al. 2000, 2003; Śliwińska et al. 2005) are encouraging and should be widely expanded to assess their general applicability. It is possible that in certain cases dry but living or subvital nuclei, if appropriately prepared, suffer to a lesser extent from cytosolic inhibitors than living leaf tissue.

Finally, a stringent and generally agreed list of standard plant species for the whole range of C-values in plant FCM has still not been achieved and should be worked out. This requires the concerted work of several laboratories, similar to the work carried out by Doležel et al. (1998). There is presently only one suitable organism whose genome has been sufficiently sequenced to serve as a general gold standard: the worm *Caenorhabditis elegans* "Bristol N2" with certified 96.893 Mbp (0.9907 pg) per 1C, which amounts to an estimated total of 100 Mbp (Bennett et al. 2003). In the chicken and human there are still uncertainties as to the precise absolute genome size (cf. Table 18.2). For *Arabidopsis thaliana*, 1C = 157 Mbp or 0.1605 pg presently seems to be the best estimate (Bennett et al. 2003).

Acknowledgments

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