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An employment of flow cytometry into plant biosystematics

J a n   S u d a

2004

P h D .   T h e s i s

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Dedicated to my parents, grandmother and my wife Radka ...

... they know why ...

T H E S I S   C O N T E N T

S U M M A R Y

This thesis is a culmination of my more than three-year work (2000-2003) in the field of flow cytometry. The principal task was to establish the technique in the Institute of Botany, Academy of Sciences, Průhonice and in the Department of Botany, Charles University in Prague. This aim was coupled with an endeavour to identify new and promising domains where flow cytometry should be particularly beneficial, and to disseminate gained knowledge among other colleagues and students.

The first part of the PhD. thesis summarizes general information about flow cytometry and its utilization in current plant biosystematics. Sources of errors and a short guide for reliable data acquisition are presented. The chapter includes also topics that are believed to represent future 'hot-spots' in plant-flow-biosystematics. The second part contains a set of eight articles (incl. one popularization) demonstrating an efficient application of flow cytometry in various branches of plant biosystematics.

Ploidy levels in *Vaccinium* sect. *Oxycoccus* were estimated in the Paper I. Subsequent morphometric analyses identified both species- and cytotype-specific characters that allowed to propose an unbiased and robust taxonomic treatment of the group. Comprehensive cytotype screening at fine spatial scale was performed in the Paper II. Sympatric occurrence of various ploidy levels was found to be quite a common phenomenon in cranberry populations, bringing further support for taxonomic concept deduced in the previous work.

Flow cytometry proved successful in the detection of rare cytotypes, as documented by a revelation of a new triploid plant (presumably of hybrid origin) in the genus *Empetrum* (Paper III). Research targeted on cytotype distribution pattern continued in the Paper IV. Co-occurrence of various ploidy levels was repeatedly found, disproving the theory of their distinct ecological separation.

Flow cytometric study on alien plants was materialized in the Paper V. Surprising variation in DNA-ploidy levels was detected within *Reynoutria* species collected in the Czech Republic.

An attempt to untangle the puzzle of polyploid *Lamium* subg. *Galeobdolon* resulted in an extensive investigation of karyological, morphological, and isozyme variations (Paper VI). Once again, only karyologically-confirmed individuals were involved, thus the conclusions and implications are not negatively influenced by subjective plant determination.

Paper VII concentrates on nuclear DNA content estimation in Macaronesian angiosperms (the first study of DNA content variation in insular flora). Remarkable concentration of species with very small DNA amounts was revealed, suggesting that rapid insular radiation is associated with genome diminution.

Beginner-friendly information on plant flow cytometry is encapsulated in the Paper VIII (I apologize to all readers not familiar with Czech language).

LIST OF PAPERS

I. **Suda J.** & Lysák M. A. (2001): A taxonomic study of the *Vaccinium* sect. *Oxycoccus* (Hill) W.D.J. Koch (*Ericaceae*) in the Czech Republic and adjacent territories. – *Folia Geobotanica* **36/3**: 303-319.

II. **Suda J.** (xxxx): Sympatric occurrences of various cytotypes of *Vaccinium* sect. *Oxycoccus* (Ericaceae). – *Nordic Journal of Botany* **22/5**: xx-xx. (in press).

III. **Suda J.** (2002): New DNA ploidy level in *Empetrum* (Empetraceae) revealed by flow cytometry. – *Annales Botanici Fennici* **39/2**: 133-141.

IV. **Suda J**., Malcová R., Abazid D., Baňas M., Procházka F., Šída O. & Štech M. (xxxx): Cytotype distribution pattern in *Empetrum* (Ericaceae) at various spatial scales in the Czech Republic. – *Folia Geobotanica* **39/2**: xx-xx (accepted).

V. Mandák B., Pyšek P., Lysák M. A., **Suda J.**, Krahulcová A. & Bímová K. (2003): Variation in DNA-ploidy levels of *Reynoutria* taxa in the Czech Republic. – *Annals of Botany (London)* **92/4**: 265-272.

VI. Rosenbaumová R., Plačková I. & **Suda J.** (2004): Variation in *Lamium* subg. *Galeobdolon* (Lamiaceae) – insights from ploidy levels, morphology and isozymes. – *Plant Systematics and Evolution* xx: xx-xx (in press).

VII. **Suda J.**, Kyncl T. & Wildová R. (2003): Nuclear DNA amount in Macaronesian Angiosperms. – *Annals of Botany (London)* **92/1**: 153-164.

VIII. **Suda J.** (xxxx): Co se skrývá za rostlinnou průtokovou cytometrií [What does the plant flow cytometry mean]. – *Živa* xx: xx-xx (accepted) [in Czech]

G L O S S A R Y

C-value

DNA amount in the unreplicated haploid nucleus, irrespective of the ploidy level of the organism (expressed either in picograms or megabase pairs; 1 pg = 978 Mbp). Resting somatic cells thus posses 2C DNA amount. In plant kingdom, the values vary from about 0.1 pg to 127.4 pg.

Coefficient of variation (CV)

An attribute of a histogram peak describing its width (expressed as a percentage). It is defined as the standard deviation divided by the mean of a series of fluorescence values, and allows to compare the quality of peaks located on different fluorescence channels. CV fundamentally affects the resolution of flow cytometric acquisitions - reasonably low threshold (e.g. 3 %) should be kept to guarantee reliable results.

Cytotype

Organisms with different ploidy levels (= number of basic, monoploid, chromosome sets). They can be treated as separate taxa or merged into one taxon without any autonomous rank.

DAPI

4´,6-Diamidino-2-phenylindole – nucleic acid stain with A-T preference. It is excited in UV, and emits blue fluorescence. Frequently used for ploidy level estimation, yielding histograms with very high resolution (low CVs).

DNA content, DNA amount

DNA quantity in resting somatic cells of the organism (equivalent to 2C-value).

Flow cytometry (FCM)

Method for rapid measurement of optical properties (e.g. fluorescence, light scatter) of isolated particles (cells, nuclei, chromosomes) moving singly in a narrow liquid stream. Estimation of nuclear DNA content is the most frequent application of FCM in plant biosystematics.

Fluorochrome

Chemical compound able to absorb light and then emit fluorescence of a different colour (different wavelength). Three basic types of fluorochromes are used in plant flow cytometry: intercalating (e.g. propidium iodide), A-T specific (e.g. DAPI), and G-C specific (e.g. mithramycin).

Genome size

Nuclear DNA amount in the monoploid chromosome set (in polyploids averaged). It should be calculated according to the formula: 2C-value / ploidy level. Genome size equals C-value in diploid organisms, however, is proportionally smaller in the polyploids.

Histogram

Two-dimensional graph used for a visualization of flow cytometric acquisitions. Horizontal axis describes the intensity of a recorded signal (usually fluorescence), vertical axis the number of objects with a given intensity range.

Propidium iodide (PI)

Nucleic acid stain intercalating into double-stranded DNA. It is excited with blue-green light, and emits red fluorescence. Popular fluorochrome for nuclear DNA amount determination. It binds also to double-stranded RNA, a pre-treatment with ribonuclease is thus essential.

I N T R O D U C T I O N

WHAT IS FLOW CYTOMETRY?

Flow cytometry (FCM) might be broadly defined as a method for measurement of optical characteristics (fluorescence, light scatter) of isolated particles (cells, nuclei, chromosomes) that flow one by one in a narrow liquid stream through a powerful beam of light.

The technique was originally developed for biomedical applications (rapid counting and analysing of blood cells) in the 1960s - 1970s (Doležel 1997a). With a construction of user-friendly and versatile flow cytometers during the last two decades, and an invention of new fluorochromes, the method has spread into various fields of biological sciences. Currently, FCM is routinely used in clinical diagnostics, biotechnology, and basic and applied research (immunology, molecular biology, genetics, pharmacology, zoology, marine biology, and botany). The power of this technique lies both in the wide range of parameters that can be simultaneously recorded and in the information on how these parameters are distributed within the particle population. By using an adequate approach, flow cytometry facilitates determination of various characteristics at cellular (size, shape, granularity, membrane potential, cell cycle, apoptosis) and intracellular levels (DNA and RNA contents, base composition, protein content, intracellular pH, calcium concentration, chromosome size, centromeric index, etc.) (Doležel 1997b, Rieseberg et al. 2001). The number of applications is further multiplied in more advanced systems equipped with a sorting unit that offers rapid separation of a defined subpopulation of particles (Doležel et al. 1995).

INSTRUMENTATION

Typical flow cytometer (Fig. 1) consists of five basic operational units (Ormerod 1999):
1) light source (laser or arc lamp)
2) flow chamber and fluidic system
3) optical assembly (lenses, filters, mirrors)
4) signal processing part (photodetectors, converters)
5) computer part

Light sources

Two types of light sources are used in commercial flow cytometers: lasers and/or arc lamps.

Lasers produce a stable, bright, narrow beam of monochromatic light. The wavelength depends on a filled gas in the plasma tube. Current plant cytometers are most commonly equipped with air-cooled argon ion lasers tuned at 488 nm (turquoise). Helium-neon lasers (emitting most light at 633 nm), red diode lasers (635 nm), green solid state lasers (532 nm) or helium-cadmium lasers (with ultraviolet excitation at 325 nm) are often available in more sophisticated systems. Spectral purity of the emitted light eliminates the need for excitation filters, however, it simultaneously represents one of the major limitations. If other wavelengths are required, another laser must be used (although some state-of-the-art water-cooled lasers can be tuned to various wavelengths). High purchase price is an additional laser disadvantage.

Mercury (or rarely xenon) arc lamps are employed as an inexpensive source of light in some bench-top flow cytometers. In plant sciences, they are well suited particularly for ploidy level estimation. Low cost and easy maintenance rank among their principal advantages over lasers. They emit non-monochromatic light, therefore a set of excitation optical filters must be used to select required wavelength (usually in UV). Mercury lamps also suffer from rather low lifetime (generally between 200 – 400 hours) and a low output power (insufficient sensitivity to analyse a weak fluorescence). Moreover, gradual decrease in light intensity within a working day can occur.
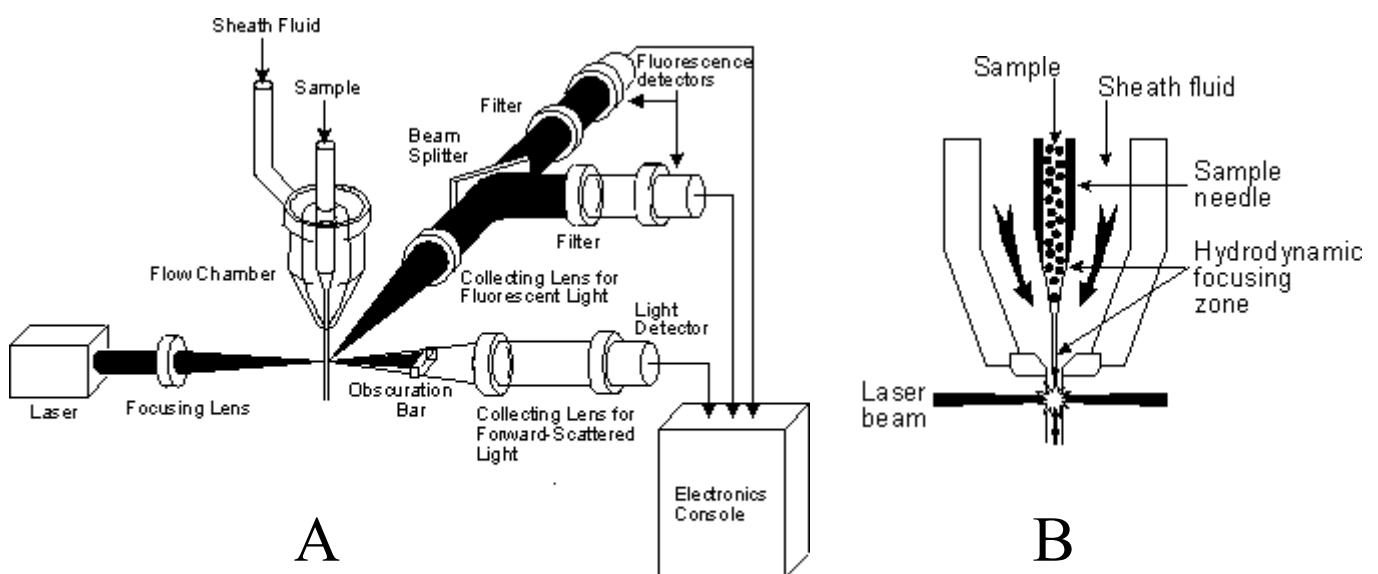

Figure 1. Schematic view of a flow cytometer (A) and a close up of the flow chamber (B) (adapted after Doležel 1997b).

Flow chamber

Flow chamber (= flow cell, nozzle) represents a central part of the instrument (Fig. 1). Its mission is to adjust the measured particles in a narrow central stream and to deliver them one after another into a focal point of the light source. This is achieved by so-called hydrodynamic focusing (Doležel 1991). The sample is injected into a stream of sheath fluid (mostly water or saline solution) moving with a greater velocity and thus confining the sample within a central core. The jet then passes through a narrowing flow chamber orifice. Induced acceleration forces the particles to move singly, and they are delivered to the point of excitation (with an accuracy of about 1 μm). Typical stream velocity is between 1 and 10 m/s that corresponds to several dozens or hundreds of analysed particles per second.

There are three basic types of flow chamber configuration (Doležel 1997b). In 'jet-in-air' design, the stream exits from the flow chamber into open air where the particles intersect a light beam. As the flow chamber orifice has a small diameter (typically 75 μm), high speed is attained. This configuration is used in most cell sorters. 'Enclosed stream' design operates with lower velocity (higher sensitivity is often achieved), and the particles are measured during their movement in a narrow capillary tube (of about 250 μm in cross-section). This arrangement is employed e.g. in Partec flow cytometers that are commonly used in laboratories working on plant material. In 'jet on open surface' design, the particles are analysed in a stream flowing on a glass coverslip.

Optical assembly

An optical part of the instrument provides focusing the excitation beam, selection of required wavelengths, collection of output light and its delivery to the detectors.

Non-adjusted laser beam has a circular shape of approximately 1-2 mm in diameter (Ormerod 1999). However, to fulfil the requirement for identical illumination of each particle, it must be focused with lenses to an appropriate profile. Elliptical spot of about 60×20 μm (with longer dimension perpendicular to the sample stream) is preferred for DNA amount analysis in 'enclosed stream' cytometers; spherical beams are often used in 'jet-in-air' systems.

The output light from illuminating particles (collected through the lens with a high numerical aperture) consists of various colours. This spectral mixture must be partitioned into specified wavelengths before reaching the photodetectors. An assembly of optical mirrors, dichroic mirrors, and colour filters is used in this mission. Standard filters accommodated in flow cytometers are the short-pass ones (transmit light below a specified wavelength), long-pass filters (transmit light above a specified wavelength), and band-pass filters (transmit light over a narrow wavelength band close to the specified value; they are generally used immediately in the front of the detectors). Dichroic mirrors (beam splitters) are placed at a 45° angle in the light path and they work in a similar fashion as filters (reflect vs. transmit light of specified wavelengths).

<u>Signal processing part</u>

The light beam adjusted by the optical bench is focused onto photodetectors that convert light signal into an electrical current impulse (photodiodes are used for a strong forward scatter, photomultiplier tubes for a fluorescence and a weak side scatter). After pre-amplification and further processing (e.g. elimination of debris and electric noise), the signal undergoes a main amplification. Both linear and logarithmic amplifiers are available in commercial flow cytometers; the former should always be used for nuclear DNA content estimation, the latter are employed e.g. in immunofluorescence studies. Final stage before storing the signals, is an analogue to digital conversion. Ten bits converters yielding 1 024 channels are most commonly used in DNA measurements.

<u>Computer part</u>

Digital data are stored, visualised, and further analysed in a built-in computer. Majority of systems use a flow cytometry standard (FCS) format for data storage. On the computer screen, data are generally displayed as univariate or bivariate histograms. It is often necessary to select certain subgroup of particles before performing statistic analyses. This procedure is called gating. Once stored, the data can be subjected to various statistic procedures to extract a required information. Mean channel position, number of particles, and coefficient of variation are the basic statistics computed in univariate analyses.

BRIEF HISTORY

A flow cytometry story begins in 1934 when a construction of equipment counting red blood cells during their passage through a capillary on a microscope stage was suggested (historical data compiled mostly from Givan 2001). Although it is still a moot point whether such apparatus has ever been built, it represented the first step from the static microscopy towards flowing systems. Fifteen years later (in 1949), an instrument for the study of blood cells was developed by Wallace Coulter. The particles travel in an isotonic saline stream, and measurements are based on an increment of electrical resistance as they pass through the orifice and replace the saline solution. This technology combines several features used in current flow cytometers (rapid flow of isolated particles, electronic detection of signals, etc.). Crucial limitation of analyses operating with particles flowing through a narrow tube was a common blockage of the capillary (wider tubes could not have been used as they allowed passing two or more particles at a time). A milestone was the year of 1953 when a principle of hydrodynamic focusing was invented. The sample is injected into the centre of a faster moving stream that forces the particles to be aligned exclusively in a narrow central core. This approach combines both the need of a tight, precisely defined fluidic jet and the requirement of a capillary tube with sufficient diameter that prevents clogging. The first fluorescence-

recording cytometer with hydrodynamic focusing was constructed in Los Alamos Laboratories in 1969. At the present, flow cytometry is developing in two directions: 1) complex instruments recording many properties of diverse particles at increasingly higher rates and sensitivity; 2) 'fool-proof' (bench-top) cytometers with elementary equipment for a routine use in laboratories.

FCM AND PLANT SCIENCES

The application of flow cytometry in plant sciences has been markedly delayed in comparison with human or animal biology (Doležel 1991). Although the first article utilizing this technology appeared already in 1973 (Heller 1973), the technique has been practised only in a few laboratories until the early 1980s. The time lag was largely caused by the requirement of a single-particle suspension. As a huge majority of plant cells is incorporated into solid, three-dimensional tissues, a method for their isolation had to be developed.

Protoplasts obtained after enzymatic digestion of cell walls were of primary choice. Unfortunately, intact plasma membrane is not permeable for DNA-specific fluorochromes, and ethanol / acetic acid fixation was essential for their sufficient penetration (Doležel 1991). Moreover, another difficulties were encountered during cytometric acquisitions. They arose from a presence of cytoplasmatic DNA, various inclusions (e.g. starch grains), high content of natural fluorochromes (e.g. chlorophyll), or non-identical position of the nucleus (due to the shift induced by a large vacuole) (Doležel 1991, Galbraith 1989).

The above-mentioned obstacles can be eliminated in analyses operating with isolated nuclei. It was demonstrated that protoplast lysis in the presence of non-ionic detergents yields intact nuclei suitable for flow cytometry (Puite et Ten Broeke 1983). Although this approach was routinely employed in several laboratories, it still suffered from laborious sample preparation. A breakthrough in plant flow cytometry emerged in 1983 when David Galbraith and his co-workers presented a simple and rapid method for mechanical isolation of nuclei from plant tissues (Galbraith et al. 1983). The tissue is chopped with a sharp razor blade in a glass petri dish containing hypotonic buffer supplemented with a non-ionic detergent and a fluorochrome. Up to now, this technique has remained by far the most frequent method for sample preparation in plant flow cytometry.

FCM ANALYSIS OF NUCLEAR DNA CONTENT

Without any doubt, analysis of nuclear DNA amount is the most widespread application of FCM in plant biosystematics. Background of DNA content estimation lies in the recording of relative fluorescence of isolated nuclei after their staining with a fluorochrome. As the particle (nucleus-fluorochrome complex) intersects the illuminating beam, several physical processes occur (Watson 1991):

1) absorption, diffraction, refraction and reflection of the incidental light
2) fluorescence (= emission of longer wavelengths when electrons fall from the excited state to the ground state)

Diffraction is generally related to the size (more precisely to the cross-sectional area) of the object. After blocking the direct beam (with a narrow obscuration bar), any light scattered at low angles is collected by a photodiode and further processed. This signal is commonly referred to as forward scatter (FSC).

The light scattered at higher angles (refraction and reflection become increasingly important) is more related to structural features of the particle such as granularity or surface irregularity. For convenience, the measurements are made orthogonal to the incidental beam and the signal is referred to as side scatter (SSC, right-angle light scatter). However, neither FSC nor SSC have found wide utilization in biosystematic studies of vascular plants.

An attention of 'flow-botanists' is almost exclusively fastened on the fluorescence. After an absorption of illuminating light, electrons in the fluorochrome molecule move to a higher energy level. The excited state is, however, not stable and the electrons fall promptly back to their ground state, releasing a heat and a fluorescent light. Due to the loss of some energy as heat, the emitted light is always of longer wavelength (and less energy). This difference (so-called Stokes shift) facilitates discrimination between the illuminating and the fluorescent lights (Givan 2001).

FLUOROCHROMES

Fluorochromes are chemical compounds able to absorb light and then emit fluorescence of a different colour (different wavelength). DNA-specificity and stoichiometric binding are essential properties to be employed into analyses of nuclear DNA content. The fluorescence intensity in free form is weak, however, it increases markedly after their binding to nucleic acids. Three groups of fluorescent stains can be distinguished with respect to the binding mode (Doležel 1991): 1) dyes quantitatively intercalating into double-stranded nucleic acids; 2) dyes selectively binding to adenine-thymine-rich regions; 3) dyes selectively binding to quanine-cytosine-rich regions. A synopsis of the most frequently used fluorochromes for estimation of nuclear DNA amount in plants is presented in the Table 1.

Table 1. Major fluorochromes used in plant flow cytometry and their binding mode to nuclei acids (after Doležel 1991).

Fluorescent stain	Binding mode	Excitation*	Emission*
Propidium iodide (PI) Ethidium bromide (EB)	Intercalation	Blue-green	Red
DAPI DIPI	A-T specific	UV	Blue
Hoechst 33258 Hoechst 33342	A-T specific	UV	Blue
Mithramycin Chromomycin A3 Olivomycin	G-C specific	Violet-blue	Green

* Actual spectral properties (exact wavelengths) depend on several factors, including DNA/dye ratio, ionic strength and acidity of the staining solution

Intercalating dyes (PI, EB) must be used when estimating nuclear DNA content in absolute units (picograms or megabase pairs). They both are usually excited by a 488 nm argon laser, although this wavelength is far from the optimal condition and hits no more than 5 percent of the excitation capacity (M. Steinberg 2003, pers. comm.); lower resolution is a penalty for that suboptimal excitation. As the dyes bind also to double-stranded RNA, a pre-treatment with ribonuclease is essential. Final concentration of intercalating dyes should be above the saturation level (recommended 50 – 70 μg / ml), with the pH of the buffer between 7.2 and 7.4 (pH below 7 results in enhanced nuclease activity) (Bennett et Leitch 2003). At the present, propidium iodide is preferred to EB due to its slightly higher resolution and lower toxicity.

Fluorochromes with A-T specificity are mostly used for ploidy level estimation. They all have similar optical properties – an excitation in UV (e.g. with mercury arc lamp) is followed by an emission of blue light. A comparative study between DAPI and Hoechst dyes revealed higher fluorescence intensity and better resolution for the former one (Otto et Tsou 1985). It is plausible that DAPI staining is less affected by the state of chromatin condenzation than is staining with other fluorochromes (Shapiro 1995). Owing these features, DAPI has received widespread usage in current plant flow cytometry (generally at concentration of 4 μg / ml).

G-C specific fluorescent stains constitute a group of closely related antibiotics with maximum absorption at about 400 nm. Final concentrations between 50 – 100 μg / ml are recommended and a presence of magnesium ions in the medium is indispensable to form a complex with DNA. Nevertheless, their impact on current plant flow cytometry is rather low, largely due to the fact that they produce histograms with lower resolution (higher coefficients of variation) than A-T specific fluorochromes (Ulrich et al. 1988).

DATA OUTPUT AND INTERPRETATION

Cytometric data from one parameter analysis (the most common type in plant biosystematic studies) are visualized in the form of 2-D histogram (Fig. 2). Horizontal axis describes the intensity of a recorded signal (usually fluorescence), vertical axis the number of objects with a given intensity range.
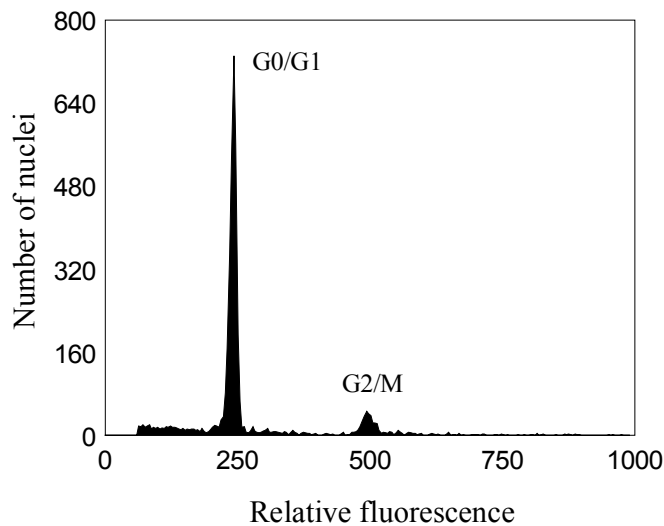
Figure 2. Typical histogram obtained after flow cytometric analysis of DAPI-stained nuclei isolated from *Anthoxanthum alpinum* (in arbitrary units). Prominent peak corresponds to the nuclei in G0/G1 phase (with 2C DNA content), smaller peak corresponds to the nuclei in G2/M phase (with 4C DNA content). Signals spanning the range correspond to the nuclei synthesising DNA (S phase). Some background fluorescence (noise) appears on low channels.

Nuclear DNA content of each eukaryotic cell undergoes cyclic changes. Resting cells (in G0 phase of the cell cycle) and cells in the primary growth period (G1 phase) have 2C nuclear DNA content (i.e. posses two copies of nuclear genome). A synthesis of new DNA occurs during S phase, resulting in doubled (4C) nuclear DNA content. In the secondary period of cell growth (G2 phase), the DNA content is maintained at 4C level. Mitosis (M phase) is terminated by a formation of two daughter cells, each with 2C nuclear DNA content.

The DNA flow histogram reflects the proportion of cells (nuclei) in particular phases. An ideal histogram would contain only a prominent peak corresponding to G0/G1 nuclei, smaller peak corresponding to G2/M nuclei, and some S nuclei spanning the range. Actually, certain variation is always introduced into FCM analyses as a consequence of both non-identical sample preparation and instrument instability. Wider peaks and additional

fluorescence signals (noise or background caused by damaged particles, non-specific fluorescence or clumped objects) are the typical symptoms.

An accuracy of FCM analysis is described by the coefficient of variation (CV) of the peak (defined as the ratio of standard deviation to the mean). It facilitates comparing the quality of peaks located on different fluorescence channels. In contrast to standard deviation, CV is generally not influenced by the peak position (Watson 1992). The CV of nuclei in G0/G1 phase mostly varies from 1 % to 10 % depending on plant material (species with low DNA content generally yield higher CV), fluorochrome used (DAPI generally gives better histograms) and the sample preparation. Nevertheless, only cytometric acquisitions with sufficiently low CVs should be accepted to guarantee reliable data interpretation. The resolution of FCM histogram critically depends on the CV: assuming identical peak heights in simultaneous analysis, CV lower than half of the difference between DNA contents of the analysed samples is imperative to achieve their discrimination (e.g. CV equivalent to 3 % would detect 6 % difference in DNA content).

ADJUSTMENT AND STANDARDIZATION

Instrument adjustment has a significant effect on the histogram quality. An accuracy of measurements should be regularly monitored using FCM calibration standards, i.e. biological or artificial particles with a homogenous fluorescence. Stained trout red blood cells and fluorescent microbeads are routinely used in plant DNA flow cytometry (both give CVs around 1 – 1.5 %). Optimal operational conditions yield maximum signal amplitude, minimal CV and minimal background.

In addition to the peak quality, a linearity of measurements should also be controlled (so that the modal position of G2/M peak is exactly twice that of G0/G1 peak). A tendency of some FCM standards to form clusters (doublets, triplets, etc.) is an appropriate feature for such inspection.

FCM estimation of nuclear DNA content always requires an involvement of a reference standard (= material with known DNA amount). Ploidy level or genome size of the analysed plant is determined by a comparison between standard – sample peak positions. Two different types of standardization exist: external and internal (Doležel 1997). In the former approach, both standard and sample are prepared and measured separately. Relative peak position of the reference standard is checked before or after each sample run. However, external standardization may considerably bias the accuracy of measurements due to instrument instability or non-identical staining conditions. On that account, it should be completely excluded from plant flow cytometry. Above-mentioned errors can be eliminated by internal standardization when nuclei from both the reference standard and the sample are isolated, stained, and analysed simultaneously. One internal standard is usually employed,

although a secondary reference point may enhance the sensitivity (especially when aneuploidy is suspected) (Pfosser et al. 1995). Plant of the same species with known chromosome number (usually diploid) is generally used as a standard for the ploidy level estimation; several different plant species (with known 2C-values) are available for the determination of genome size. The following reference standards have been successfully employed in our lab: *Raphanus sativus* L. cv. Saxa (2C = 1.11 pg), *Lycopersicum esculentum* Mill. cv. Stupnické polní tyčkové rané (2C = 1.96 pg), *Glycine max* (L.) Merrill cv. Polanka (2C = 2.5 pg), *Zea mays* L. cv. CE-777 (2C = 5.43 pg), *Pisum sativum* L. cv. Ctirad (2C = 9.09 pg), and *Vicia faba* L. (=*Faba vulgaris* Moench) cv. Inovec (2C = 26.9 pg) (Doležel et al. 1992, Doležel et al. 1994, Lysák et Doležel 1998). Some other reference standards have also been recommended (Johnston et al. 1999), however, they mostly posses large genomes and thus do not sufficiently cover the entire range of plant DNA contents.

An accurate determination of nuclear DNA amount should follow some basic rules (particularly when measured in absolute units):

1) small ratio between 2C-values of the analysed plant and the internal standard to minimise potential non-linearity of FCM measurement

2) avoiding very close or overlapping peaks of the analysed plant and the internal standard

3) accepting only analyses yielding peaks of approximately the same height (to eliminate bias due to greatly different number of particles); at least 5 000 particles should be recorded

4) using the same internal standard for all taxa belonging to one genus (or the group examined) to facilitate a comparative study

5) re-estimation of absolute nuclear DNA content at least three times on different days (by the same operator and the same instrument)

Moreover, herbarium vouchers kept in public herbaria should be available for the material being measured by FCM, and each novel 2C-value should be supplemented with a chromosome count.

Setting minimal difference between peak positions of the internal standard and the sample to 12 percent, recommended standards for plant material with given 2C-value ranges are presented in the Table 2.

Table 2. Internal reference standards recommended for determination of absolute nuclear DNA amount in samples with given 2C-value ranges (orig.).

Sample 2C-value (pg)	Recommended reference standard
0 – 0.98, 1.24 – 1.47	*Raphanus sativus* (2C = 1.11 pg)
0.98 – 1.24, 1.47 – 1.72, 2.20 – 2.80	*Lycopersicum esculentum* (2C = 1.96 pg)
1.72 – 2.20, 2.80 – 3.68	*Glycine max* (2C = 2.50 pg)
3.68 – 4.78, 6.08 – 7.03, 8.00 – 10.18	*Zea mays* (2C = 5.43 pg)
4.78 – 6.08, 7.03 – 8.00, 10.18 – 15.64, 23.67 – 30.13	*Pisum sativum* (2C = 9.09 pg)
15.64 – 23.67, more than 30.13	*Vicia faba* (2C = 26.90 pg)

ADVANTAGES OF FCM

Flow cytometry has several important advantages over laborious chromosome counting:

The sample preparation is very easy and convenient (i.e. chopping and chopping ☺), and it lasts only few minutes. Even the 'most time-consuming' two-step procedure involving a centrifugation does not exceed ten minutes. The particles are measured at high speeds, several dozens per second being usually an optimal rate. Owing to the rapidity, numerous samples can be investigated within one working day.

High accuracy of FCM assays facilitates detection of minute variation in nuclear DNA amount. Differences as low as four percent have been manifested in several plant species in our laboratory.

Non-destructiveness is another feature of a paramount importance. Only small quantities of plant tissue (e.g. 0.5 cm^2) are required for the sample preparation. This opens a way for comprehensive studies of rare and endangered plant species without a risk of their population destruction or decline. An investigation of seedlings in very early ontogenetic stage is also feasible.

Although young leaves are most frequently used for nuclei isolation, a variety of other plant tissues can be utilized, i.e. roots, stems, sepals, petals, or seeds (it seems that just juicy fruits do not yield satisfactory histograms). In contrast to conventional karyological studies, mitotically active cells are not indispensable for FCM acquisitions. This advantage will be undoubtedly highly appreciated by everybody whose attempts to find mitoses in microscopic slides were in vain.

FCM also represents an ideal tool to detect endopolyploidy and / or mixed samples. As a fluorescence of each particle is recorded separately, two or more peaks will be recognized in that kind of material. High accuracy to identify minority cytotype (even at proportion of about 10 %) has been repeatedly demonstrated in various plant species (Suda et al., unpubl.). This property makes the technique very attractive for rapid cytotype screening.

Last but not least, low operating costs should also be underlined (unfortunately, this is not applicable to the instrument). Remarkable and often unexpected results can be gained even with a low budged (very common condition in Czech science).

LIMITATIONS OF FCM

Like any other analytical method, FCM suffers from some limitations. A requirement of fresh plant material is a true Achilles heal that precludes its comfortable utilization in field botany. Since the samples should be analysed as soon as possible (even turgor decrease might deteriorate the histogram resolution), commuting from the field to the lab and vice versa has become an integral part of 'flow-botanist' life. Fortunately, majority of plant species can be stored fresh in the refrigerator at 4 ºC for several days. This time can be prolonged up to several weeks in taxa possessing tough and leathery leaves. Some attempts to employ fixatives (ethanol or ethanol / acetic acid) have also been made. Hülgenhof et al. (1988) demonstrated the feasibility of isolated nuclei fixation, however, a tendency to clumping was a concomitant phenomenon. Moreover, mechanical isolation of intact nuclei from formaldehyde-fixed tissues was reported by Sgorbati et al. (1986). Although further targeted work is essential, this approach may significantly contribute to the enhancement of FCM versatility. Ploidy level estimation of herbarium vouchers that has recently been initiated in our lab (Suda, in prep.), also seems very promising (see Future perspectives).

Conventional karyological studies outcompete flow cytometry in assays operating with aneuploid plants or individuals possessing accessory (=B) chromosomes. A chance to detect single chromosome variation by FCM is limited to species having less than about 25 chromosomes (so that a volume of one chromosome equals approximately 4 %).

Nuclei isolation and staining poses serious problems in some vascular plants. These are often associated with a presence of tannins (e.g. in *Geraniaceae*, *Rosaceae*, conifers), mucilaginous compounds (e.g. in *Betulaceae, Violaceae, Lythraceae, Malvaceae*, submerged plants), or high concentration of organic acids (e.g. in *Crassulaceae* and other groups with CAM metabolism). Samples prepared from leaf tissue of mature plants in some *Boraginaceae* (e.g. *Echium*) yield hardly any fluorescence signal, although this limitation disappears when young seedlings are measured (Suda, unpubl.).

RELATED TECHNIQUES

In contrast to biochemical techniques that generate pooled signal for the whole particle population, values peculiar to every single object are registered in flow cytometry. Identical approach has been utilized also in other related methods. As these techniques operate with particles fixed on a solid surface (not moving in a flow), they are in summary called static cytometry. Two principal categories can be distinguished: fluorometry (DNA amount is estimated from the fluorescence intensity, similarly to FCM) and densitometry (DNA amount is deduced from the optical density of the stained region) (Vilhar et al. 2001).

The latter techniques are based on DNA staining with the Feulgen reaction. The optical density can be measured either by a microscope photometer (i.e. microdensitometry, microspectrophotometry) or, in more sophisticated systems, by an automated computer image analysis (i.e. image cytometry). Here, the images are grabbed from the microscope via a video or digital camera and the optical density is calculated from the grey values of pixels in the nucleus (Vilhar et al. 2001). Although rather fragmentary data on plant biosystematic applications are still available, image cytometry has received an increased attention recently (e.g. Dimitrova 1999). In addition to DNA content estimation, the technique also offers a possibility to examine several other particle attributes such as size, morphology, number, etc. A fixation of plant material is another advantage over FCM, although this is compensated by more laborious sample preparation.

Fluorometric techniques are represented by a cytofluorometry and a laser-scanning cytometry. The former is based on the recording of emitted light by a microscope with incorporated photometer. However, as cytofluorometry can not compete with FCM either in speed or convenience, its application is currently on decline. On the contrary, laser-scanning cytometry is a state-of-the-art method that combines unique attributes of both flow and image cytometries (Darzynkiewicz et al. 1999). Nevertheless, its astronomical price still hinders more widespread usage.

APPLICATIONS OF FLOW CYTOMETRY IN CURRENT PLANT BIOSYSTEMATICS

Estimation of nuclear DNA content (either in relative or absolute units) is without any doubts the most frequent application of FCM in modern plant biosystematics. This approach facilitates rapid screening of ploidy level, determination of genome size, or convenient detection of reproduction mode.

PLOIDY LEVEL ESTIMATION

Polyploidy has played a significant role in the evolution of vascular plants - up to 80 % of angiosperms and about 95 % of pteridophytes were estimated to be polyploid (Leitch et Bennett 1997). It is unlikely to come as any surprise that FCM has found a large-scale utilization in plant sciences.

Ploidy level screening

The knowledge of ploidy level provides a reliable criterion for taxa determination in numerous taxonomically intricate groups of vascular plants. Representative examples from Central-European flora are *Veronica hederifolia* agg. with three microspecies (2x, 4x, 6x), or *Galium mollugo* agg. and *Polypodium vulgare* agg. comprising each two taxa. The microspecies from cited alliances differ only negligibly in morphological characters, however, they are unambiguously defined by unique number of chromosomes (ploidy level). Hybrid identification in these groups is far beyond the limits of traditional morphological approach, and ploidy level estimation represents an essential approach for their unbiased detection. Unfortunately, there is still an appreciable deficit of that kind of studies, although I believe that flow cytometry owns a great capacity to elucidate taxonomy in various polyploid complexes.

On the contrary, much information has been amassed on FCM utilization in breeding programmes of various crops. Industrial applications of flow cytometric ploidy analysis cover (Doležel 1997b):
- screening for haploid plants (e.g. regenerants from anther or ovary cultures)
- screening for diploid plants (e.g. spontaneously occurring dihaploids)
- screening for triploid plants (e.g. hybrids with low seed production)
- screening for higher polyploids (e.g. individuals with high biomass production)
- screening for interspecific hybrids
- assessing the ploidy purity of seed lots

Few examples where rapid screening for suitable germplasm has recently been materialized include *Medicago sativa* (Brummer et al. 1999), *Acacia* (Blakesley et al. 2002), *Citrus* (Tusa et al. 2002), or *Oxalis tuberosa* alliance (Emshwiller 2002). It has been documented that the rise of ploidy is often associated with an expression of apomixis (Naumova et al. 1999, Bantin et al. 2001, Quarin et al. 2001). Under artificial conditions, the polyploidization is usually induced by a colchicine treatment and suitable mutants are subsequently selected using FCM that can easily cope with high numbers of samples. Similarly, this technique has been routinely employed in the horticulture in order to identify novel or perspective cytotypes, such as in *Dahlia* (Gatt et al. 1998), *Primula* (Kato et Mii 2000), or *Viola* (Ajalin et al. 2002). In addition, flow cytometry has proved successful in ploidy estimation of *in vitro* cultures that are generally characterized by a low mitotic activity precluding conventional chromosome counting (Winkelmann et al. 1998, Thiem et Śliwińska 2003). An ensuing rapid screening of regenerants has also been extensively practised (Vagera et al. 1994, Brutovská et al. 1998).

Aneuploidy and sex detection

In contrast to routine inspection of ploidy modification, a detection of aneuploidy (especially when only one chromosome is involved) is much more ambitious task. Nevertheless, it has been documented that the sensitivity of FCM is sufficient to detect a presence of a pair of rye chromosomes in wheat-rye addition lines (Bashir et al. 1993) or aneuploid individuals in triticale (Pfosser et al. 1995). Promising results as to the tentative identification of aneuploid plants in *Hieracium* subg. *Pilosella* were also achieved in our laboratory.

An existence of sex chromosome heteromorphism in some dioecious plants provides an opportunity for male and female separation even at very early stage of their ontogenesis. Costich et al. (1991) demonstrated the feasibility of such task using 'indirect' sex comparison; individuals of both sexes were processed separately and their DNA contents were subsequently compared. Further improvement arose in the study of Doležel et Göhde (1995) who carried out simultaneous analyses. They showed that females in both *Melandrium album* and *M. rubrum* have lower DNA content than males, and precise FCM assays would thus yield two non-overlapping peaks. However, extremely high resolution is a prerequisite for that kind of analyses due to minute differences in DNA amount between sex chromosomes (small X and large Y).

Endopolyploidy, somatic mosaic, agmatoploidy

Endopolyploidy (=endoreduplication, recurrent duplication of DNA without mitosis) is a common phenomenon in various eukaryotic organisms (Nagl 1976). Flow cytometry represents in all probability the most convenient technique for reliable detection of that type of genome organization.

Traditionally, it has been believed that endoreduplication is associated with very small genomes, assuming a certain minimum amount of DNA necessary for proper cell functions. Recent investigations on vascular plants, however, revealed only low negative correlation between genome size and degree of polyploidization (Barow et Meister 2003). On the contrary, the authors suggested that taxonomic position is the major component determining the endopolyploidy, while life cycle, genome size and organ type have only a minority, supporting effect. *Brassicaceae* (e.g. Galbraith et al. 1991) and succulent plants from various families (De Rocher et al. 1990, Mishiba et Mii 2000) seem to be more susceptible to endoreduplication. For example, somatic tissues of young cabbage (*Brassica oleracea*) seedlings exhibited up to 16-fold increase of nuclear DNA content (= 4 rounds of DNA synthesis without cell division) compared with diploid complement present in resting embryos (Kudo et Kimura 2001a), and even higher DNA amount was observed in mature flowers (Kudo et Kimura 2001b). An increase of DNA content is frequently connected with tissue differentiation to a specialized function; storage organs such as endosperm (Kowles et al. 1997), cotyledons (Bino et al. 1993), or pod walls (Lagunes-Espinoza et al. 2000) are the illustrate examples. The degree of endoreduplication in particular organs might be influenced by ploidy level as indicated by significant differences between diploid and tetraploid cytotypes of maize (Biradar et al. 1993). Remarkable phenotype manifestation of elevated DNA amount was described in cultivated azaleas (*Rhododendron* subg. *Tsutsusi*) (De Schepper et al. 2001). Variegated flowers with broad, differently coloured petal edges proved to be somatic mozaic – tetraploid in the edges, diploid in the rest of petals. Gene dosage effect and increased cell size were proposed to be responsible for that kind of flower coloration.

Agmatoploidy (non-lethal chromosome fragmentation owing to the presence of diffuse centromere) is a common feature of two angiosperm families (*Juncaceae, Cyperaceae*). This process results in the increase of chromosome number (either proportional to the haploid set or not) under constant nuclear DNA amount. Simultaneous employment of conventional karyological counting and flow cytometry is thus obligatory for reliable identification of agmatoploid individuals.

Population cytotype structure

Cytotype distribution over vast geographic areas provides useful insight into population biology of multi-ploid taxa. However, large-scale cytotype investigations had been enormously time-consuming until flow cytometry was routinely employed (Lewis et al. 1967). Fortunately, papers based on 1 500 plus individuals are far from the utopia nowadays (e.g. Burton et Husband 1999). This progress allows to raise numerous challenging questions of current biosystematics, such as the overall distribution pattern (sympatry vs. parapatry), investigation of evolutionary forces governing the cytotype co-existence, ecological preferences of individual cytotypes, inter- vs. intra-cytotype competition, or mechanisms of reproductive isolation (Petit et al. 1999).

Large single cytotype areas were found to be a common feature in several species, *Plantago media* (van Dijk et al. 1992), *Cham(aen)erion angustifolium* (Husband et Schemske 1998), and *Themeda triandra* (Liebenberg et al. 1993) being some examples. Contact zones harbouring two or more cytotypes were very narrow in these taxa and generally did not exceed few kilometres. On the contrary, cytotype mixing over large geographic areas was encountered e.g. in *Pennisetum* sect. *Brevivalvula* (Renno et al. 1995). A co-existence of more cytotypes within a single population undoubtedly deserves particular attention. These populations are most frequently constituted by diploids and their autotetraploid derivates, as in *Artemisia* subg. *Tridentatae* (McArthur et Sanderson 1999) or *Centaurea jacea* (Hardy et al. 2000). Nevertheless, an intermingling of different polyploids has also been documented: hexaploid and enneaploid individuals grew in natural populations of *Andropogon gerardii* (Keeler et Davis 1999), tetraploids and hexaploids predominated in *Buchloë dactyloides* (Johnson et al. 1998). The phenomenon of different cytotype co-existence can be explained either by 1) a directional selection or 2) a balanced selection (Weiss et al. 2002). The former theory assumes that the cytotype intermingling is a transitional stage and one cytotype will be finally outcompeted (minority cytotype exclusion); the latter type of selection assumes that the co-existence of different cytotypes can be maintained for a long time due to the development of various isolating mechanisms. Flowering time divergence observed in *Arrhenatherum elatius* (Petit et al. 1997) and in *Dactylis glomerata* (Bretagnole et Thompson 1996), or microhabitat differentiation in *Anthoxanthum alpinum* (Felber-Girard et al. 1996) are the examples of prezygotic barriers reducing potential production of hybrids and thus favouring cytotype co-existence. Similarly, a 'triploid block' promoting intra-cytotype mating has evolved in several diploid-tetraploid groups (Hardy et al. 2001).

NUCLEAR DNA CONTENT DETERMINATION

C-value and genome size

Nuclear DNA amount is an important feature of all living organisms. DNA content in the unreplicated haploid nucleus (irrespective of the ploidy level of the taxon) is referred to as C-value (Swift 1950). DNA content of the monoploid chromosome set (in polyploids averaged) is referred to as genome size. C-value thus equals genome size in diploid species, however, always exceeds genome size in polyploid species. Both C-value and genome size can be expressed either in DNA picograms ($=10^{-9}$ g) or megabase pairs (1 pg = 978 Mbp; Doležel et al. 2003).

Remarkable variation in C-values has been found in plant kingdom (Bennett et Leitch 2003): 1C-values range from about 0.16 pg in *Arabidopsis thaliana* to 127.4 pg in *Fritillaria assyriaca*, differing about 800-fold. Although even smaller values (e.g. 0.05 pg in *Cardamine amara*) have been reported, they are bound to be erroneous and were not confirmed by

subsequent critical investigations. A theoretical minimum for vascular plants is believed to be about half of the *Arabidopsis* genome (M. Bennett 2003, pers. comm.). Increasing need for C-value data and their rapid and easy accession led to the publication of several summarizing lists covering data for approx. 1.4 % of the total angiosperm flora (Bennett et Smith 1976, 1991, Bennett et al. 1982, 2000, Bennett et Leitch 1995, 1997). Moreover, the C-value database(s) currently harbouring estimates for 3 927 land plant species are available in electronic form at Royal Botanical Gardens web page (Bennett et Leitch 2003). This cumulative approach allowed to determine major gaps in our knowledge of nuclear DNA amount and propose principal goals for the forthcoming period. Particular attention during last few years has been paid to better coverage of plant families. Several targeted works were published (Hanson et al. 2001a, b, 2003), nevertheless, the C-values are still unknown for about 50 percent of angiosperm families (with more data for monocots and basal angiosperms and less for eudicots). Similarly, additional information on nuclear DNA content is urgently needed for pteridophytes, as no more than 82 reliable estimates (from approx. 9 000 species) are available (Bennett et Leitch 2001, Obermayer et al. 2002). Much more satisfactory situation is encountered in gymnosperms where familiar representation has been completed (Leitch et al. 2001). Mosses have generally been neglected in comparison with vascular plants (Renzaglia et al. 1995, Temsch et al. 1998) until the appearance of precisely targeted investigation of Voglmayr (2000) who estimated nuclear DNA amounts for 138 different moss taxa representing all major clades (except *Sphagnum*). Basic descriptive statistics on C-values for all major groups of extant plants are summarized in the Table 3.

Table 3. Basic descriptive statistics on 1C-values for major groups of living plants (data taken from Bennett et Leitch 2003, revised).

	Mean	Lowest value	Highest value	Mode	Median	Range	Number of taxa
Bryophytes	0.5	0.17	2.05	0.45	0.43	c. 12	171
Pteridophytes	11.5	0.16	72.7	7.95	7.95	c. 450	78
Gymnosperms	17.1	2.25	32.2	9.95	16.25	c. 14	265
Angiosperms	6.34	0.16	127.4	0.55	2.9	c. 800	3 485

Information on C-value and genome size has been fruitfully utilized in various branches of plant sciences. Taxonomists undoubtedly appreciate the fact that some related species with identical number of chromosomes might differ in DNA volume, being thus easily recognizable using FCM. Dimitrova et al. (1999) investigated Bulgarian populations of *Crepis foetida* and concluded that - despite the karyotype constancy - subsp. *commutata* posses about

10 % smaller genome size than the nominate subspecies and subsp. *rhoeadifolia*. Similarly, almost five-fold range of variation was documented in the genus *Hypochaeris*, with highly significant differences among nearly all species (Cerbah et al. 1999). Species-specific C-values were obtained in *Colchicum* (Fridlender et al. 2002), several taxa or sections could also have been delimited in *Helleborus* (Zonneveld 2001), *Hydrangea* (Cerbah et al. 2001) or *Lilium* (Siljak-Yakovlev et al. 2003). The power of genome size data lies also in the opportunity to detect hybrid individuals from homoploid crosses, providing the putative parents differ enough in nuclear DNA amount (generally at least about eight percent). Flow cytometric identification of hybrid plants in *Dryopteris dilatata* alliance is currently being under way in our laboratory (Fig. 3).
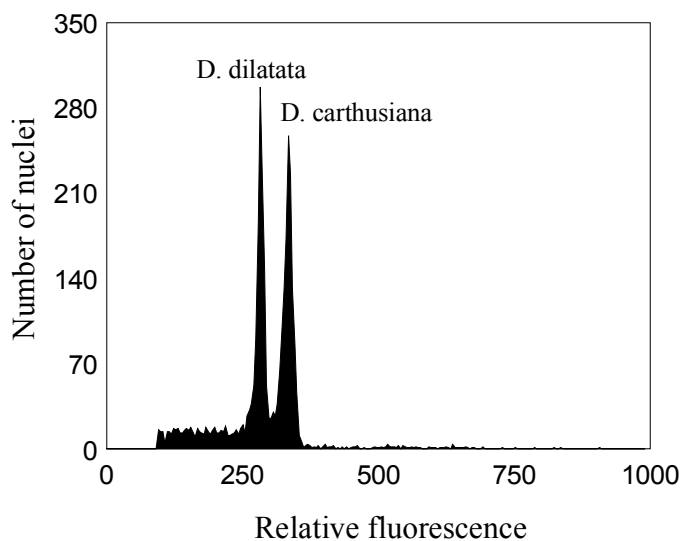
Figure. 3. Flow cytometric profile of two species from *Dryopteris dilatata* alliance with identical number of chromosomes (2n = 164) but different nuclear DNA amount.

Another fascinating domain is an assessment of genomic constitution in allopolyploid taxa (=polyploid plants combining genomes from at least two different parental species). For example, wheat genome is constituted from three different components (A, B, D), and the D genome (2C = 5.05 pg) seems to contain markedly less DNA than both A (2C = 6.15 pg) and B (2C = 6.09 pg) genomes (Lee et al. 1997). Lysák et al. (1999) demonstrated about 12 percent difference between genomes A (donated from *Musa acuminata*) and B (donated from *M. balbisiana*) present in triploid banana cultivars, and proposed that comparative analysis of genome size in diploids and triploids may be helpful in identifying putative diploid progenitors of cultivated triploid *Musa* clones. Our laboratory is engaged in similar project aimed to elucidate species relationships in *Hieracium* subg. *Pilosella* on the basis of different

C-values of particular taxa. The group under investigation is a typical example of taxonomically intricate polyploid complex whose structure is substantially influenced by hybridization.

The genome size and C-value may also be beneficial in predicting various phenotypic characters, or phenological and ecological behaviour. For this reason, C-values should be treated as a fundamental factor involved in the scaling of living systems (Bennett et al. 2000a). Positive correlations were found between DNA amount and nuclear and cell volumes, mitotic cycle duration, and duration of meiosis (Van't Hof et Sparrow 1963, Evans et Rees 1971). The same relationships manifested at tissue and organ levels involve, among others, seed weight and volume (Kenton et al. 1986, Chung et al. 1998). An interest in C-values extends also to ecological and environmental issues. Genome size significantly affects minimum generation time in herbaceous plants: ephemerals, on average, have the smallest genomes, followed by annuals, whereas obligate perennials have higher DNA amounts (Bennett 1972). Negative correlation between nuclear DNA content and the probability of being recognized as weedy species is thus not surprising (Bennett et al. 1998). Analogously, variation in plant phenology (Grime et Mowforth 1982, Grime et al. 1985), or the sensitivity to frost (MacGillivray et Grime 1995) may be related to genome size. It has been demonstrated that species with small genomes tend to grow later in the year than species with large genomes, and are more susceptible to late-frost events. Development in early spring involves mainly sudden expansion of cells formed during previous summer, while growth later in the season is predominantly realized by quick cell divisions. Significant negative correlation between genome size in *Allium* and the first month of flowering (Baranyi et Greilhuber 1999) corroborates the concept. Moreover, *Allium* species with the highest DNA amount have long dormant period, whereas only very short winter dormancy is typical for species with the lowest DNA amount (Ohri et al. 1998). Not only temperature, but also other environmental factors may be mirrored by genome size. Diploids with lower DNA content in the genus *Berberis* grew in sites with greater rainfall but lower water availability (Bottini et al. 2000), and negative relationship between precipitation and genome size was observed among 18 North American *Pinus* species (Wakamiya et al. 1993). Nevertheless, presented correlations with ecological or climatic variables are certainly not a general property of all plant groups, and no equivalent relationships have been found in numerous other taxa (e.g. Palomino et Sousa 2000).

Another scientific field with practical utilization of C-value data is a modern molecular research. It has been demonstrated that species with large genomes (C-value above 20 pg) may cause problems when investigating by standard AFLP procedure (Bennett et al. 2000a). An illustrative example is *Alstroemeria* (Han et al. 1999) possessing C-values from ca. 18 pg to 39.5 pg (Buitendijk et al. 1997). Protocol modification involving either change in restriction enzymes or in the number of selective bases was recommended for these taxa.

Valuable information has been extracted when inspecting C-values data in the phylogenetic context (superimposing DNA values on the robust consensus phylogenetic tree) (Leitch et al. 1998). It was demonstrated that ancestral angiosperms most probably possessed very small C-values (1.4 pg or less), whereas very large C-values (35 pg and more) represent a derived condition that evolved at least twice, in monocots and in higher eudicots (namely family *Santalaceae*).

Noteworthy accumulation of angiosperms with very small C-values in the Macaronesia has recently been revealed in our laboratory (Table 4; see also Paper VII). Representatives of Macaronesian endemic genera crown the pattern, and 19 out of 23 genera investigated (82.6 %) posses very small DNA amounts. Interestingly, only limited variation in 1C nuclear DNA content among endemic plants was ascertained, ranging from 0.19 pg in *Descurainia bourgeauana* to 9.52 in *Scilla dasyantha* (about 50-fold difference). It is plausible that the selection for small genomes is coupled with rapid angiosperm diversification on islands (adaptive radiation), and with requirements for fast individual development. The theory of very small genomes preference may also be helpful in the explanation of very low abundance of DNA-obese geophytes in the Macaronesia.

Table 4. Marked difference in proportions (%) of angiosperms with particular C-values in the Macaronesia and in the rest of the world (data on non-Macaronesian species taken from Bennett et Leitch 2003, revised)

	very small (≤ 1.4 pg)	small (1.41 – 3.5 pg)	intermediate (3.51-14 pg)	large (14.01-35 pg)	very large (> 35 pg)
Macaronesian (n = 241)	67.22	20.33	12.45	0	0
Non-Macaronesian (n = 3 478)	32.29	23.29	32.26	10.46	1.70

Base composition

In addition to nuclear DNA content, the base composition (proportion of AT / GC base pairs) yield further information about genome organization (Schwencke et al. 1998, Dagher-Kharrat et al. 2001, Ellul et al. 2002, Siljak-Yakovlev et al. 2002). The base composition obviously differs among plant families (for example, *Poaceae* have relatively small AT frequency), but usually exhibits high similarity at lower taxonomic levels (Barow et Meister 2002).

Base-specific fluorescent dyes (often DAPI with AT preference) are employed in that kind of investigations. Although DAPI is being popular in FCM arena for more than two decades, the exact nature of its binding to DNA still remains rather speculative. Originally,

linear correlation between DAPI fluorescence and AT base proportion has been expected. Nevertheless, Godelle et al. (1993) reappraised that premise and derived a formula based on a curvilinear relationship between DAPI fluorescent intensity and AT content. They assumed that certain number of consecutive bases of the same type is necessary to bind a dye molecule (e.g. 3-4 bases were proposed for DAPI, 5 bases for Hoechst 33342). Final algorithm for calculation of AT bases proportion is as follow:

AT (%) sample = AT (%) standard $\times$ (DAPI/PI)$^{1/n}$, where
    DAPI – ratio between standard / sample peak positions in DAPI staining
    PI – ratio between standard / sample peak positions in PI staining
    n – number of consecutive bases, usually set to 4

Although presented formula has been widely employed into the estimation of base composition, recent investigations revealed several inconsistencies. They were attributed either to the effect of non-randomness of base sequences on dye binding (Barow et Meister 2002) or to the incorrectness of simple theory of several consecutive bases (Meister 2003). Measurements performed by the latter author indicated that the binding length for all investigated dyes is near to 1, and in no way greater than 2. Similar discrepancies as well as rather fluctuating results when compared the base composition in various species pairs were experienced also in our laboratory. In accordance with Barow et Meister (2002), I thus incline to the résumé that '... in general, a good approximation of AT content can be computed on the basis of the DAPI factor, but important deviations are possible in some cases.'

REPRODUCTION MODE SCREENING

A revolutionary advance in FCM methodology represents an efficient method for reproduction mode screening published recently by Fritz Matzk and his co-workers (Matzk et al. 2000). They brilliantly linked flow cytometry with the knowledge of different proportional DNA content of embryo and endosperm in mature seeds, depending on the reproductive pathway. Considering the type of male and female gametes (reduced vs. unreduced), the embryo origin (zygotic vs. parthenogenetic), and the endosperm origin (pseudogamous vs. autonomous route), ten different pathways of seed formation can be reconstructed. The novel approach combines several advantages such as speed, convenience and low cost, and yields reliable information on reproductive behaviour useful in various fields of plant sciences. It has soon been adopted in plant breeding and taxonomy as a powerful tool for reproduction mode characterization (Cáceres et al. 2001, Heenan et al. 2002). Model histograms obtained in our laboratory demonstrating different profiles in sexual and apomictic individuals are shown at Fig. 4.
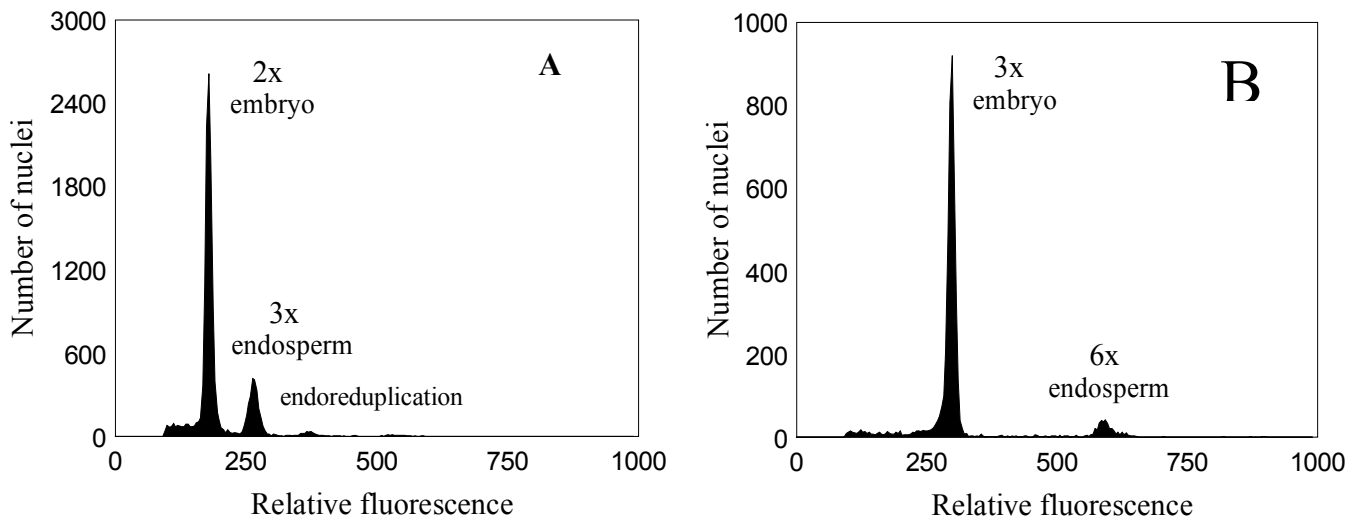
Figure 4. Flow cytometric histograms obtained from DAPI-stained nuclei isolated from mature seeds of *Taraxacum* spp. - obligate sexual individuals (A); obligate diplosporous apomicts with autonomous endosperm formation (B). Additional small peaks represent endoreduplication events.

DOUBLE CHECK BEFORE YOU PUBLISH

Publish or perish – a motto of today's science has accelerated a pernicious trend to write papers by the yard irrespective of their reliability. Numerous erroneous and dubious results can be located in cytometric literature. It is a good idea to discard all FCM articles demonstrating minute variation in nuclear DNA content if the authors:

1) used only external standardization
2) did not perform sufficient number of replicates on different days
3) did not provide compelling arguments that potential negative effect of secondary metabolites had been avoided
4) did not achieve reasonably low coefficients of variance; a representative histogram should also be presented
5) did not confirm the variation in simultaneous analyses

The following table summarizes some examples of artefactual variation together with the author(s) who exposed the blunder or doubted the original data. Papers were selected irrespective of the technique employed; both densitometric (mostly Feulgen densitometry) and fluorometric (mostly FCM) methods are included.

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Table 5. A list of selected articles demonstrating intraspecific variation in nuclear DNA content that was, however, disproved by subsequent investigations (based mostly on Greilhuber 1998).

Taxon	Original paper(s)	'Blunder-killing' paper(s)
Arachis hypogea *A. duranensis*	Singh et al. (1996)	Temsch et Greilhuber (2000, 2001)
Collinsia verna	Greenlee et al. (1984)	Greilhuber (1998) based on Greenlee et Rai (1986)
Dasypyrum villosum	Cremonini et al. (1994) Frediani et al. (1994)	Obermayer et Greilhuber (2003)
Festuca arundinacea	Ceccarelli et al. (1992, 1993)	Greilhuber (1998) very probably erroneous
Glycine max	Graham et al. (1994) Rayburn et al. (1997)	Greihuber et Obermayer (1997) Greilhuber (1998) Obermayer et Greilhuber (1999)
Hedera helix	Kessler et Reches (1977) Schäffner et Nagl (1979)	König et al. (1987)
Helianthus annuus	Nagl et Capesius (1976) Olszewska et Osiecka (1983) Cavallini et al. (1986, 1989, 1996) Arumuganathan et Earle (1991) Michaelson et al. (1991) Johnston et al. (1996) Price et Johnston (1996) Price et al. (1998)	Greilhuber (1998) Price et al. (2000)
Linum usitatissimum	Evans et al. (1966) Evans (1968a,b) Durrant et Jones (1971) Joarder et al. (1975) Cullis (1985)	Greilhuber (1998) very probably erroneous, proposed to re-estimate using flow cytometry
Pinus spp.	Berlyn et al. (1990)	Greilhuber (1998)
Pisum sativum	Cavallini et Natali (1990, 1994) Cavallini et al. (1993)	Greilhuber et Ebert (1994) Baranyi et Greilhuber (1995, 1996)
Sambucus racemosa (and other species with 'floral DNA')	Nagl et al. (1979) Nagl et al. (1983)	Greilhuber (1988b)
Scilla siberica	Deumling et Clermont (1989)	Greilhuber (1998)
Vicia faba	Ceccareli et al. (1995) Minelli et al. (1996)	Greilhuber (1998) both very probably erroneous

The given table is by no means exhaustive, and additional suspicious cases can be traced even in modern literature (e.g. Wyman et al. 1997, Serrato-Cruz et al. 2000). Obvious

accumulation of some authors (e.g. Cavallini, Ceccarelli, Cionini, Minelli, Natali) supplies an evidence that non-optimal method has been constantly adopted in their laboratory. Therefore, any other results should also be treated with caution and should not be cited uncritically. Ohri (1998) summarized additional examples of intraspecific variation reported in angiosperms. However, as numerous records on that kind of variation have broken down to nothing when appropriate methods were applied, it would be a good idea to re-analyse the same material using the best techniques currently available (particularly those taxa that has become textbook examples, such as *Micromeria* (Price et al. 1980, 1981a, b)).

A presence of secondary metabolites ranks among important triggers of artefactual variation. Much knowledge on the deteriorating effect of secondary compounds has been accumulated for the Feulgen staining (Greilhuber 1988a, b). Polyphenol substances (particularly tannins) occur commonly in some groups of vascular plants. In intact cells, they are trapped in vacuoles, however, they are releasing during the sample preparation. As polyphenols bind strongly to the chromatin (and proteins), they interfere with nuclear dyes and cause significant reduction of staining intensity. Similar effect can be induced even by low-molecular weight compounds such as catechin or quercetin (common flavonoid).

Flow cytometric analyses are bound to be negatively influenced in the same way. For example, Noirot et al. (2000) demonstrated that cytosolic components may markedly affect fluorochrome accessibility to nuclear DNA. They noted up to 20% decrease in fluorescent intensity of petunia nuclei after an addition of nuclei-free extract from yam (*Dioscorea alata*) leaves. Similarly, reported intraspecific variation in DNA content in sunflower (*Helianthus annuus*) leaves can be most simply explained by a variation in the amount of inhibitory compounds (Price et al. 2000). These nucleus-cytosol interactions clearly highlight the sensitivity of chromatin structure (e.g. condenzation intensity) to extranuclear factors.

A taxonomic criterion must also be taken into consideration when assessing intraspecific genome size variation. *Scilla bifolia* s.l. can be chosen as an instructive example (Greilhuber et Speta 1985). When this group is treated as a single species, it shows about two-fold genome size variation. Nevertheless, the variation disappears after its splitting up into relatively inconspicuous, but natural taxa.

Although I do not *a-priori* reject the existence of intraspecific variation, I strictly believe that this phenomenon is certainly much less common than previously thought. The theory of genome size constancy has gained numerous supporting pieces of evidence recently. For example, any significant differences were observed neither among wheat near isolines differing in aluminium tolerance (Wetzel et al. 1999), nor among disjunct populations of

Abies fraseri (Auckland et al. 2001), and *Sesleria albicans* (Lysák et al. 2000). Remarkable degree of stability in nuclear DNA content was also demonstrated in *Allium cepa* cultivars originating from four continents (Bennett et al. 2000b). Nevertheless, taxa adapted to various climates, occurring in diverse habitats over vast geographic area, spatially isolated autogamic races or crops under long-lasting human selection might potentially manifest some difference in nuclear DNA content. Maize (*Zea mays*) may be considered as a model crop plant displaying variation in C-value. This variation (up to 37 %) was shown to correlate with the size and number of heterochromatic knobs (Laurie et Bennett 1985, Rayburn et al. 1985). Maize behaviour corresponded well with the nucleotype theory (Bennett 1972) – races having smaller genomes (and thus more rapid development) were better adapted to the growth in more northerly regions. Allopolyploid species with multiple origins represent another candidates where intraspecific nuclear DNA content variability may be detected.

BEST PRACTICE FOR NUCLEAR DNA CONTENT ESTIMATION USING FLOW CYTOMETRY

Although basic rules for reliable estimation of nuclear DNA content were presented in the introductory chapters, they will be summarized here once again. I believe that as long as these rules are followed, the artefactual variation could be eliminated from FCM measurements:

1) internal standardization should always be used

2) nuclear DNA contents of the internal standard and the sample should be reasonably close (but not overlapping or extremely close)

3) the same internal standard is recommended for all taxa belonging to the group examined. If more internal standards are necessary (to span the range of DNA amounts), the secondary standard must be calibrated against the primary one (it is not a good idea to use tabulated values).

4) young (but not premature), intact, parasite- and disease-free leaves of both sample and internal standard should be used. The plants should be cultivated under the same conditions to eliminate potential negative effect of different levels of secondary metabolites.

5) intercalating propidium iodide (+ RNAse) at concentration between 50 – 70 μg / ml (pH 7.2 – 7.4) must be chosen as a fluorochrome for DNA content determination in absolute units (more sensitive DAPI with A-T preference provides only expression in relative values)

6) each measurement must be repeated at least three times on different days (repetitions on the same day are useless). If the variation between individual runs exceeds some critical value (personally, I mostly use two-percent threshold),

additional measurements should be performed. The most out-layered measurement can be avoided.

7) the peaks of both the internal standard and the sample should be symmetrical (non-skewed) and of approximately the same height. The fluorescence of at least 5 000 particles should be recorded.

8) the analyses must not be distorted by the presence of secondary metabolites – the mean peak position of the internal reference standard should be located on (almost) identical fluorescence channel in all measurements, either with or without the sample

9) the coefficient of variation of G0/G1 peaks should be reasonably low (generally below 3 %, although higher values can be regarded as acceptable e.g. in plants possessing very small nuclear DNA content)

10) all measurements must be performed on the same flow cytometer with the same light source of excitation, as small but systematic differences exist between different types of equipment (Doležel et al. 1998); the same operator is also recommended. The cytometer should be adjusted to maximum signal amplitude, minimal CV and minimal background. In Partec intruments, the gain (signal amplification) should not exceed the value of ca. 600 (ideally less than 500).

11) it is advisable to measure at least three individuals per taxon to inspect potential intraspecific DNA amount variability. In wide-range studies, this point can be substituted by a realization of simultaneous analysis of several (usually 3-5) individuals. If one narrow and symmetrical peak is achieved, one representative plant can be selected and further investigated.

12) exact chromosome number should be determined for the material analysed. This point has become absolutely necessary when intraspecific variation is suspected (aneuploidy or a presence of accessory chromosomes may be responsible for that kind of variation).

13) confirmation of true variability in nuclear DNA content requires additional pieces of inspection. Simultaneous analyses of samples supposed to differ in nuclear DNA content should be performed (bifurcated peak corroborates the theory of non-identical DNA amount). An employment of two reference standards (so that the DNA content of the sample is located in-between) is another advocated approach. The peak ratio of both reference standards should be (almost) the same in all analyses, either with or without the sample.

14) each estimation should be supplemented by a herbarium voucher kept in public herbarium

15) last but not least recommendation: the operator should stay cheerful all along the experimentation ☺

FUTURE PERSPECTIVES

Two different directions of FCM assays attract my attention for the future: 1) rather routine estimation of ploidy level or genome size in order to understand better the taxonomy in selected plant groups, relationships between genome size and ecological attributes, or to elucidate processes undergoing in plant populations (i.e. organism-targeted approach); 2) methodological issues that might contribute to the enhancement of FCM utilization in plant biosystematics (i.e. methodologically-targeted approach).

Among others, the former way includes ploidy level screenings in *Dryopteris dilatata* and *Arenaria serpyllifolia* alliances, a study of genome size variation at large spatial scale in *Lythrum salicaria*, or a comprehensive analysis of nuclear DNA amount variation in the genus *Anthoxanthum*. Genome size estimation in insular vascular plants (do they have smaller genomes than their mainland counterparts?), and in the Czech endemics are another tasks of a great interest. I believe that particularly fruitful will be detailed investigations of sympatric occurrence of various cytotypes, including spatial changes in their distribution pattern (e.g. in *Campanula*, *Lysimachia*, *Pimpinella*, etc.). A search for rare triploid individuals in diploid-tetraploid groups and a study of their role in microevolutionary processes also deserve special attention. These tasks provide an appropriate platform for a linkage between flow cytometry and other methods of current biosystematics, such as multivariate morphometrics, isozyme analyses, and molecular approaches.

Methodological direction covers the matters of troublesome species investigation or non-fresh material utilization, and are separately discussed below.

TROUBLESOME MATERIAL – HOW TO DEAL WITH?

Properly adjusted flow cytometer together with optimal sample preparation and suitable plant material will yield high-resolution histograms (Ulrich et Ulrich 1991). The very best analyses realized by Doležel et Göhde (1995) demonstrate that CV as low as 0.53 % might be achieved (my personal record is 0.86 % in DAPI-stained nuclei of *Pisum sativum*). Nevertheless, a number of vascular plants produce histograms that are far from our expectations (if gives any reproducible fluorescence signal). Standard procedure of nuclei isolation (i.e. leaf chopping in a hypotonic lysis buffer) largely fails in plants holding high level of mucilaginous compounds. The final suspension is very viscous and traps the nuclei that can not pass through the mesh during filtration. Phenolics (e.g. tannins) have become a nightmare in the flow cytometry arena since a revelation of their role in generating stoichiometric errors in nuclear DNA content determination (Greilhuber 1986, Greilhuber 1988a).

When run into difficulties, following steps can be taken to enhance a quality of the histogram:

1) choosing of another isolation and / or staining buffer. Our preferred method for nuclear DNA amount estimation is a two-step procedure originally described by Otto (1990). This approach gives optimal results in the majority of vascular plants. In some groups, a simplified one-step modification can be alternatively used. LB01 buffer (Doležel et al. 1989) also works well with a variety of species, although it generally yields higher CVs. Tris-MgCl$_2$ buffer (Pfosser 1995) proved to be successful e.g. in *Arabidopsis* sessions (i.e. taxon possessing very small genome). Relevant method for DNA amount determination in seeds (embryo + endosperm) was presented by Matzk et al. (2000). The histogram quality might also be significantly affected by a fluorochrome employed (DAPI generally gives better resolution than PI). Our results showed that PI-stained nuclei in *Rubus* spp. (*Rosaceae*) yielded hardly any reproducible signal in contrast to narrow peaks obtained after their staining with DAPI.

2) inclusion of antioxidants or chemical compounds preserving the nuclei integrity. 2-mercaptoethanol is being routinely added into staining buffers to avoid polyphenol oxidation (recommended concentration of 2 μl / ml). Yokoya et al. (2000) demonstrated that the presence of PVP-40 (at concentrations of at least 10 mg / ml) significantly increased the fluorescence intensity and reduced the nuclear debris in the genus *Rosa*. Without PVP-40 addition, the peaks were often undetectable due to the phenolic interference with DNA staining. Therefore, the authors supposed that the chemical assisted in maintaining the integrity of nuclear envelope. Similarly, an addition of PVP-10 (1%) into the isolation buffer considerably improved histogram quality in *Rubus* (Thiem et Śliwińska 2003).

3) selection of different plant tissue that would produce acceptable histograms (instead of commonly used leaf laminas). Table 3 presents a list of some troublesome genera investigated in our lab and tips how to cope with them. Chopping of leaf petioles or young stems is advisable in mucilaginous-rich vascular plants (see Fig. 5), shrubby legumes, or some *Crassulaceae*. Tannin-related problems can be reduced in sessions operating with seeds or seedlings. However, preliminary results indicate that seeds are not suitable for nuclear DNA amount estimation as embryo-nuclei have different staining properties than nuclei isolated from leaves (possibly a consequence of different chromatin structure; see Rival et al. 1997). The fluorescence intensity of seed-nuclei was approximately 10 – 20 percent higher than that of leaf-nuclei (observed in simultaneous analyses with leaf tissue of the internal standard e.g. in *Scilla*, *Lavatera* or *Marcetella*). Further targeted work is essential to test whether an involvement of internal standard seeds will eliminate this discrepancy. The concentration of tannins can

also be cut down in roots, although a care should be taken when interpreting the histograms due to often pronounced G2/M peak. Similarly, an employnment of etiolated leaves might be advantageous in some plant groups.

Table 6. Selected vascular plants difficult to analyse using leaf lamina – flow cytometry and the tips for histogram improvement. Any modification has not yet been successful in genera *Helianthemum* and *Geranium*.

| Species (family)	Presumable source of problems	Suggested tissue
Viola (*Violaceae*)	mucilaginous compounds	leaf petioles
Lythrum (*Lythraceae*)	mucilaginous compounds	young stems
Urticularia – submerged spp. (*Lentibulariaceae*)	mucilaginous compounds	flowering stems
Ocotea (*Lauraceae*)	mucilaginous compounds	inflorescence axes
Lavatera (*Malvaceae*)	mucilaginous compounds	cotyledons
Marcetella (*Rosaceae*)	tannins	cotyledons
Aeonium, Monanthes (*Crassulaceae*)	organic acids	stems, very young leaves
Reynoutria (*Polygonaceae*)	organic acids, tannins (only with PI)	the youngest (in-rolled) leaves
Echium (*Boraginaceae*)	?	cotyledons
Teline, Spartocytisus, Chamaecytisus (*Fabaceae*)	estimation feasible, although with rather high CVs	young stems
Helianthemum (*Cistaceae*)	mucilaginous compounds	??? (seeds only)
Geranium (*Geraniaceae*)	tannins	???
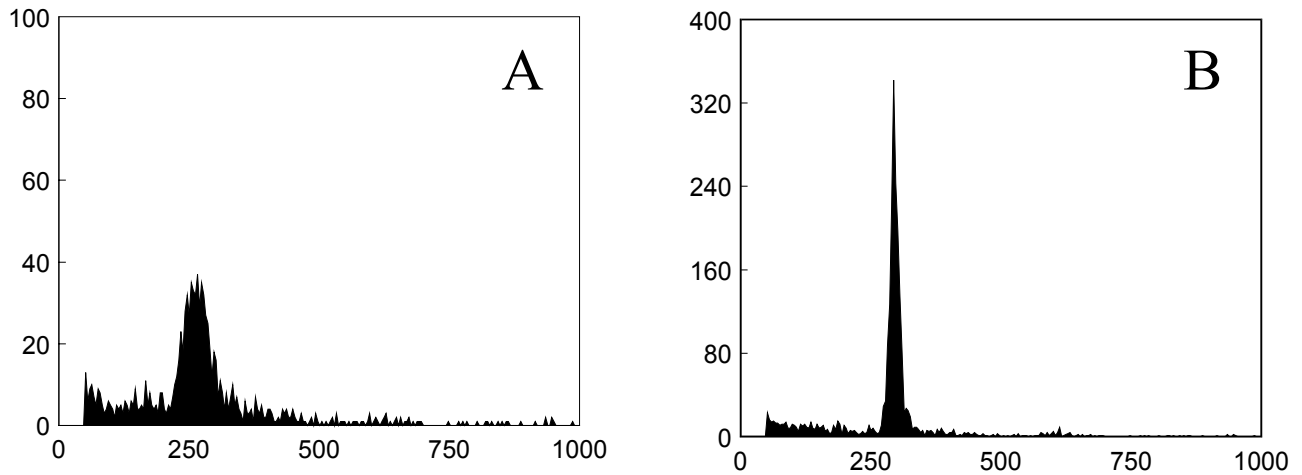
Figure 5. Comparison of histogram quality in *Lythrum salicaria* sessions using nuclei isolated from leaf (A) and stem (B) tissues.

PLOIDY LEVEL ESTIMATION IN HERBARIUM VOUCHERS

Routine analyses of non-fresh plant tissues would further multiply both the convenience and the versatility of FCM assays and give a scope for numerous additional applications in plant biosystematics. Sgorbati et al. (1986) demonstrated that formaldehyde-fixed *Pisum sativum* tissues yielded a large number of nuclei suitable for flow cytometry. The amount of nuclei released from fixed leaves and roots was even 3-12 times higher compared to the fresh material, allowing a work with much smaller tissue quantities. However, only a short-term storage of the fixed material (less than a week) was realized and the performance of nuclei after a long-term storage is still in the need of further study.

Herbarium vouchers preparation (i.e. pressing and drying) is a conventional technique for plant preservation in field botany. As plant material of immense scientific value is kept in herbaria, a method facilitating its investigation using FCM would open new prospects.

Promising results have been achieved in our laboratory using DAPI-stained nuclei isolated from herbarium vouchers of some vascular plants by both two-step and simplified one-step Otto procedures. *Vaccinium* subg. *Oxycoccus* (cranberry) comprising four cytotypes (2x, 4x, 5x, 6x) was selected as a model taxon to evaluate an impact of different drying methods (room temperature, 60 °C), different storage conditions (room temperature, deep freezer at −78 °C), and the age of herbarium vouchers on histogram quality. The present results clearly document the feasibility of ploidy level estimation in herbarium material by

FCM. Although rather short-term data are still available (the project started in 2001), some preliminary conclusions can be drawn:

- nuclei released from dry tissues show uniform fluorescence and produce peaks with reasonable CVs (e.g. as low as 2.09 % after one-year storage at room temperature; Fig. 6, Table 7).

- staining properties of nuclei remain unchanged after dehydration. Simultaneous multi-ploidy analyses of dry material always gave identical peak ratios as in fresh tissue sessions (Fig. 6). Similarly, simultaneous analyses of fresh and dry leaves of the same cytotype yielded one symmetrical peak, and the peak ratio remained stable at any combinations of fresh and dry material (e.g. fresh diploid + dry tetraploid = dry diploid + fresh tetraploid).

- diploid cytotypes generally produce peaks with higher CVs due to the interference with low-channel background signals

- the number of nuclei released from dry leaves is significantly reduced in comparison with the fresh ones; larger volume of plant tissue is therefore required for sample preparation

- drying method (room temperature / 60 °C) has no impact on the peak quality

- the accuracy of analyses is not negatively influenced by standard protective treatments in herbaria, e.g. pyrethroide gassing

- maximum life-span of herbarium vouchers remains still speculative and is largely affected by the taxon investigated. My personal record using dry leaves is a 6-year old voucher of diploid *Empetrum nigrum* (Fig. 7).

- diploid vouchers seem to have a more pronounced longevity (in the sense of FCM utilization) in comparison with their polyploid counterparts. In higher polyploids, the number of nuclei gained from dry tissues is declining more rapidly and the background signal is becoming more prominent. An investigation of older herbarium vouchers (2-5 years) with known ploidy levels showed that polyploid cytotypes often did not produce any meaningful signal in contrast to clearly defined peaks in diploid individuals of the same age.

- the longevity of herbarium samples is significantly prolonged by their storage in deep freezer at –78 °C (CVs as low as 1.81 % were achieved using one-year old leaves from *Vaccinium* sect. *Oxycoccus*). The difference in peak quality between vouchers kept at room-temperature vs. in deep-freezer was rather low after a short-time storage, however, it has been continuously increasing with the time elapsed (Table 7).

- it seems that plants to be investigated by FCM should be preferably collected and exsiccated in autumn. Summer-collected herbarium specimens gave histograms with higher CVs and more prominent background signals. Reduced level of secondary metabolites before winter hibernation might be a potential explanation. Nevertheless, a comparative study utilizing also winter- and spring-collected samples is urgently needed.
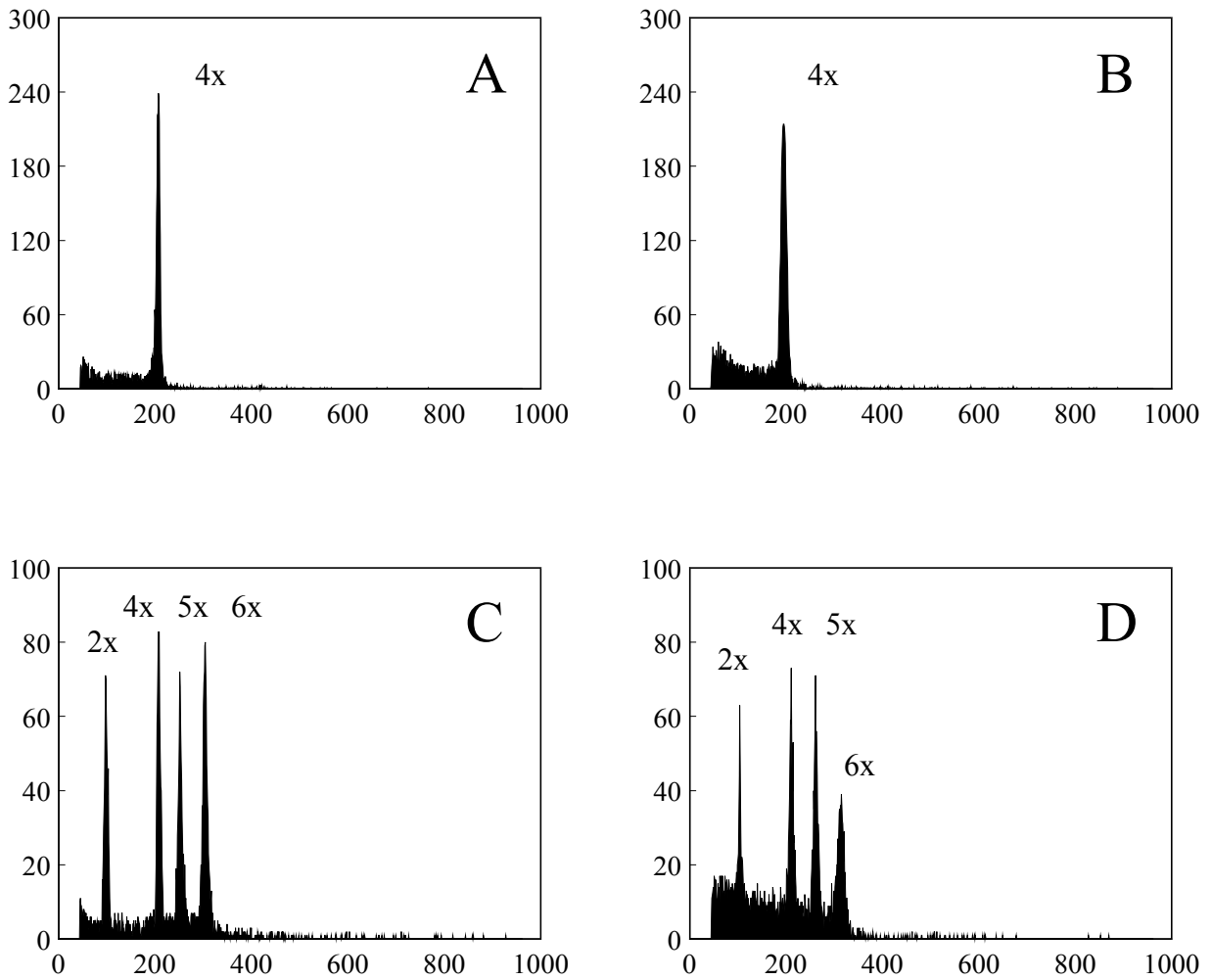
Figure 6. Flow cytometric profiles of DAPI-stained nuclei of *Vaccinium* sect. *Oxycoccus* isolated from tetraploid herbarium vouchers: one-year old (A), two-year old (B); simultaneous analysis of four cytotypes: fresh tissues (C), one-year old herbarium vouchers (D).

Table 7. Coefficients of variance (%) achieved in DAPI-FCM assays using fresh and dry (herbarium voucher) leaves of *Vaccinium* sect. *Oxycoccus*.

	Fresh	1-year old room temperature	2-year old room temperature	2-year old deep freezer
Diploid	2.66 – 3.54	3.46 – 5.91	7.51 – 9.89	6.60 – 8.08
Tetraploid	1.61 – 2.65	2.15 – 3.29	4.78 – 6.90	3.69 – 4.25
Pentaploid	1.78 – 2.55	2.09 – 3.48	5.50 – 6.41	3.36 – 3.82
Hexaploid	1.71 – 2.31	2.10 – 3.24	5.05 – 6.52	3.22 – 3.84

- seeds can be used for ploidy level determination in older herbarium vouchers instead of leaves (they generally have longer 'cytometric life-time' that may exceed a dozen of years). Although this approach seems to be very helpful, a care must be taken when interpreting the data as: 1) there is a lack of information whether seed staining properties are stable during the time; 2) nuclei of two different ploidy levels (embryo + endosperm) are present in the seed; 3) ploidy level of progenies (seeds) may differ from that of parental plant (as a consequence of hybridization, involvement of non-reduced gametes, or haploid parthenogenesis). Due to this aspect, the seed-FCM must be completely avoided in groups with versatile breeding systems (e.g. *Hieracium* subg. *Pilosella*, Krahulcová et al., in prep.) that can easily generate progenies of several different ploidy levels.

- an investigation of herbarium vouchers remains unfeasible in several vascular plants. The histogram quality varied significantly between the genera and families forming a continuum of CV values (Table 8). Unfortunately, an attempt to select any key character(s) that would predict the accuracy of FCM assays was in vain. Plants with rigid, leathery leaves, resistant to water stress are more likely to yield reproducible FCM profile; on the contrary, species rich in secondary metabolites, rapidly loosing their turgor, or possessing very small genomes are bound to produce no meaningful signal.
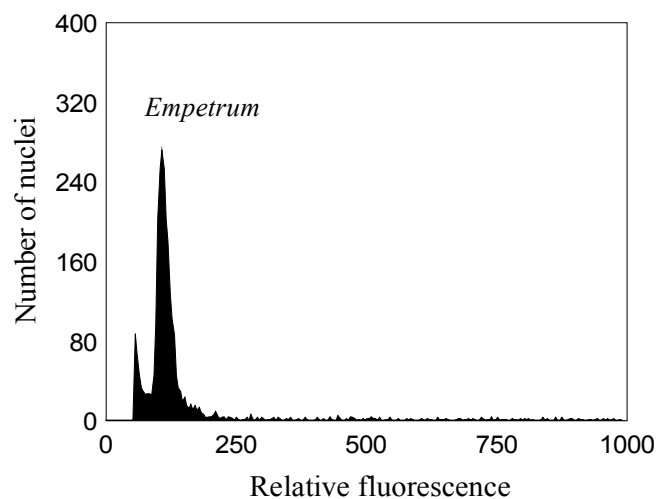

Figure 7. Flow cytometric histogram of DAPI-stained nuclei isolated from 6-year old herbarium voucher of diploid *Empetrum nigrum*.

Table 8. Coefficients of variance in selected vascular plant genera achieved in flow cytometric assays using nine-month old herbarium vouchers stored at room temperature (stained with DAPI).

Low CVs		Intermediate and high CVs		No signal
Genus (Family)	CV	Genus (Family)	CV	Genus (Family)
Polypodium (*Polypodiaceae*)	2.58	*Saxifraga* (*Saxifragaceae*)	4.04	*Fragaria* (*Rosaceae*)
Ajuga (*Lamiaceae*)	2.70	*Trifolium* (*Fabaceae*)	4.14	*Cardamine* (*Brassicaceae*)
Festuca (*Poaceae*)	2.83	*Helleborus* (*Ranunculaceae*)	4.82	*Hedera* (*Araliaceae*)
Helictrotrichon (*Poaceae*)	2.95	*Juniperus* (*Cuppressaceae*)	4.86	*Myrtus* (*Myrtaceae*)
Ilex (*Aquifoliaceae*)	3.23	*Salix* (*Salicaceae*)	5.29	*Sempervivum* (*Crassulaceae*)
Ulex (*Fabaceae*)	3.30	*Galium* (*Rubiaceae*)	5.39	*Pinus* (*Pinaceae*)
Asparagus (*Asparagaceae*)	3.34	*Doronicum* (*Asteraceae*)	6.15	*Helianthemum* (*Cistaceae*)
Luzula (*Juncaceae*)	3.34	*Thymus* (*Lamiaceae*)	7.26	
Dianthus (*Caryophyllaceae*)	3.57	*Plantago* (*Plantaginaceae*)	7.67	
Iberis (*Brassicaceae*)	3.65	*Lysimachia* (*Primulaceae*)	8.61	

NUCLEAR DNA CONTENT DETERMINATION IN POLLEN GRAINS

Gametes with sporophytic chromosome number (2n) play an important role in microevolution of several vascular plant groups. Thus, a rapid and convenient method for large-scale screening of ploidy level in pollen grains (particularly the frequency of 2n pollen formation) would significantly increase the number of current biosystematic tasks. There is still only very fragmentary information whether FCM can be widely employed in pollen investigation, and an accumulation of additional pieces of knowledge is essential.

Pollen grain analysis is hampered by the auto-fluorescence of sporopolenins or other constituents of pollen wall (often producing exaggerated noise signals), irregular shape (precluding size measurements using forward-scatter signal), and rather complicated releasing of nuclei (an improvement may be achieved using germinating pollen grains, however, this approach will introduce another bias due to potential difference in germinability between pollens with gametophytic and sporophytic chromosome numbers). In addition, special care must be taken when interpreting the histograms as bicellulate and tricellulate pollen grains produce completely different cytometric profiles (Bino et al. 1990). Above-cited limitations thus often lead to the preference of traditional examination of pollen diameter using microscope (e.g. Ortiz 1997).

Nevertheless, it has been shown that rapid pollen grains investigation using FCM is feasible (at least in some cases). Bino et al. (1990) demonstrated that DAPI was able to pass through the wall of both dry and fresh mature pollens and stain the nuclei. Subsequent chopping and filtration induced a releasing of nuclei that produced interpretable histograms (observed in *Dendrathema grandiflora*, *Lilium longiflorum*, and *Zea mays*). Similarly, DAPI-

stained nuclei isolated from pollen grains of *Petunia* yielded narrow peaks useful for DNA amount estimation (Mishiba et al. 2000). Successful identifications of unreduced pollen grains were also documented in intraspecific hybrids of *Lilium* (Van Tuyl et al. 1989), *Dactylis* (Maceira et al. 1992), and more recently in *Cupressus dupreziana* (Pichot et El Maâtaoui 2000).

OBSCURE HISTOGRAM INSTEAD OF EPILOGUE

An interpretation of some histograms is very easy, other profiles need more time to be untangled, and few analyses are still in the lap of the gods. An example of such 'hard nut to crack' is presented below (Fig. 8). Ploidy level estimation in *Dactylorhiza fuchsii – maculata* alliance often yielded three clear peaks – the prominent one corresponding to G0/G1 nuclei of *Dactylorhiza*, the small peak corresponding to G2/M nuclei, and the small peak of unknown nature located on low fluorescence channels. Several potential explanations should be excluded: 1) presence of symbiotic or parasitic organisms (fungi, rushes, etc.) – due to rather high DNA amount; 2) *Dactylorhiza* nuclei negatively affected by secondary metabolites – due to quite a low CV; 3) endopolyploidization event (so that the main peak corresponds to G2/M nuclei) – due to the peak ratios. Very similar flow cytometric profiles were observed also in the Jodrell Laboratory, Kew (M. Fey 2003, pers. comm.). On the contrary, investigation of nuclear DNA content in *Dactylorhiza* using image cytometry did not reveal any bizarre events (J. Greilhuber 2003, pers. comm.). Reliable explanation of presented phenomenon thus remains an open question. Any ideas are welcomed at my e-mail address: suda@natur.cuni.cz.
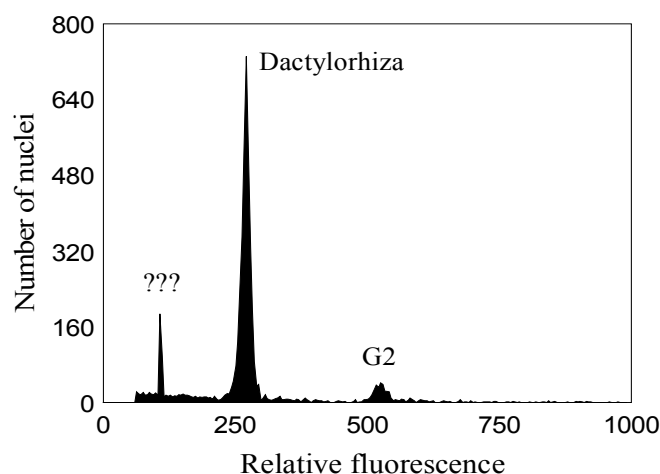

Figure 8. Histogram obtained in DAPI-stained nuclei isolated from *Dactylorhiza fuchsii-maculata*. Any attempts to interpret the nature of the small peak on the left were in vain. Do you have any illuminating idea?

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