



## Flow cytogenetics and plant genome mapping

Jaroslav Doležel<sup>1</sup>, Marie Kubaláková<sup>1</sup>, Jan Bartoš<sup>1</sup> & Jiří Macas<sup>2</sup>

<sup>1</sup>Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Sokolovská 6, Olomouc, CZ-77200, Czech Republic; Tel: (+420) 585 228 521; Fax: (+420) 585 228 523; E-mail: dolezel@ueb.cas.cz; <sup>2</sup>Laboratory of Molecular Cytogenetics, Institute of Plant Molecular Biology, Branišovská 31, České Budějovice, CZ-37005, Czech Republic

**Key words:** chromosome-specific DNA libraries, chromosome sorting, flow cytometry, genomics, physical mapping

### Abstract

The application of flow cytometry and sorting (flow cytogenetics) to plant chromosomes did not begin until the mid-1980s, having been delayed by difficulties in preparation of suspensions of intact chromosomes and discrimination of individual chromosome types. These problems have been overcome during the last ten years. So far, chromosome analysis and sorting has been reported in 17 species, including major legume and cereal crops. While chromosome classification by flow cytometry (flow karyotyping) may be used for quantitative detection of structural and numerical chromosome changes, chromosomes purified by flow sorting were found to be invaluable in a broad range of applications. These included physical mapping using PCR, high-resolution cytogenetic mapping using FISH and PRINS, production of recombinant DNA libraries, targeted isolation of markers, and protein analysis. A great potential is foreseen for the use of sorted chromosomes for construction of chromosome and chromosome-arm-specific BAC libraries, targeted isolation of low-copy (genic) sequences, high-throughput physical mapping of ESTs and other DNA sequences by hybridization to DNA arrays, and global characterization of chromosomal proteins using approaches of proteomics. This paper provides a comprehensive review of the methodology and application of flow cytogenetics, and assesses its potential for plant genome analysis.

### Introduction

Twenty years before the publication of this review, in March 1984, a paper by de Laat & Blaas (1984) marked the beginning of plant flow cytogenetics. The authors described the preparation of a suspension of intact chromosomes from a model plant, *Haplopappus gracilis* ( $2n=4$ ), and the separation of its two chromosome types by flow cytometry. At this time, flow cytogenetics was still a young science, but was already having

a notable impact on human genetics, and showing the first promising results in farm animals (Van Dilla *et al.* 1986, Grunwald *et al.* 1989).

Flow cytogenetics is the use of flow cytometry and sorting for classification and purification of chromosomes isolated from mitotic cells (Carrano *et al.* 1983). The analysis is based on the ordered flow of metaphase chromosomes at high speed in a narrow liquid stream through a beam of intense light. Pulses of scattered light and

fluorescence of stained chromosomes are quantified, and the analysis (called flow karyotyping) facilitates classification of large populations of chromosomes according to their size, morphology and relative fluorescence intensity. Individual chromosomes may be purified at high speed by breaking the liquid stream into droplets and deflecting electrically charged droplets containing chromosomes of interest by a passage through an electrostatic field (Givan 2001).

Hopes that flow karyotyping could be used for automatic detection of numerical and structural chromosome aberrations in humans (Boschman *et al.* 1992) have remained largely unfulfilled. On the other hand, flow cytometric sorting turned out to be invaluable for the purification of large quantities of wild-type and aberrant chromosomes whose DNA could be used in applications such as physical gene mapping, isolation of molecular markers, preparation of painting probes, and construction of chromosome-specific DNA libraries. Up to the present, there has been no substitute for flow cytometry when large quantities of chromosomes are needed and/or preparation of high-molecular-weight DNA is required.

These applications appeared to be attractive to plant geneticists. Compared with *Arabidopsis* and rice, the two plant species whose genomes have been sequenced, most crop plants possess much larger genomes whose size is many times greater than that of the human genome. In addition, the polyploid nature of many crops represents an obstacle to their analysis. Fractionation of their genomes into defined parts, such as chromosomes and chromosome arms, could facilitate gene mapping and cloning. However, as in many other cases, plant cytogenetics had to struggle with characteristics inherent in plants, and methods developed for humans and animals were not easily transferable to plants. The two main problems were the preparation of suspensions of intact chromosomes and the discrimination of individual chromosome types, a prerequisite for their sorting (Doležel *et al.* 1994). The purpose of this paper is to review the twenty years of development of plant flow cytogenetics, the applications of flow-sorted plant chromosomes, and to assess the potential and prospects of flow cytogenetics for plant genome mapping.

## Methodology

The procedure for chromosome analysis and sorting using flow cytometry consists of cell cycle synchronization and accumulation of mitotic cells in metaphase, preparation of chromosome suspensions, and their analysis and sorting (Doležel *et al.* 1999b).

### *Choice of material*

Three biological systems were considered as sources of plant chromosomes for flow cytometry. Suspension-cultured cells appeared attractive as large populations of cells could be easily manipulated. This was the system used by de Laat & Blaas (1984) and subsequently also by Arumuganathan *et al.* (1991) and Wang *et al.* (1992) to isolate chromosomes of tomato and wheat. The fact that the cultures may be heterogeneous (Arumuganathan *et al.* 1991), karyologically unstable (Leitch *et al.* 1993, Schwarzacher *et al.* 1997), and may be difficult to initiate in other species, stimulated a search for other experimental systems. A French group pioneered the use of leaf mesophyll protoplasts (Conia *et al.* 1987, 1989). As the frequency of metaphase cells was rather low, and because, for many plant species, protoplast culture is unsuccessful or unreliable, this system has not been used further. In 1992, Doležel *et al.* (1992) showed that suspensions of intact chromosomes could be prepared from root tip meristems, which are karyologically stable. This system has been universally accepted as it may be easily modified for use with various species (Table 1). The use of genetically transformed 'hairy' root cultures is an option when specific cytogenetic stocks cannot be propagated sexually (Veuskens *et al.* 1995, Neumann *et al.* 1998).

### *Induction of mitotic synchrony*

Suspension-cultured cells could be synchronized by nutrient starvation and transfer to fresh medium (Arumuganathan *et al.* 1991). Similarly, a transfer to culture medium induced synchronous division of leaf mesophyll protoplasts (Conia *et al.* 1987, 1989). As the degree of synchrony depends critically on the state of the cultured

Table 1. List of plant species from which flow cytometric analysis of mitotic chromosomes has been reported.

Species	Material	n*	Number of discriminated chromosomes		References
			Standard karyotype**	Cytogenetic stock***	
<i>Avena sativa</i>	Root meristems	21	0	1 <sup>a</sup>	Li <i>et al.</i> (2001)
<i>Cicer arietinum</i>	Root meristems	8	5	—	Vláčilová <i>et al.</i> (2002)
<i>Haplopappus gracilis</i>	Suspension cells	2	2	—	de Laat & Blaas (1984), de Laat & Schel (1986)
<i>Hordeum vulgare</i>	Root meristems	7	1(2)	3	Lysák <i>et al.</i> (1999), Lee <i>et al.</i> (2000)
<i>Lycopersicon esculentum</i>	Suspension cells	12	0	—	Arumuganathan <i>et al.</i> (1991)
<i>Lycopersicon pennellii</i>	Suspension cells	12	2	—	Arumuganathan <i>et al.</i> (1991, 1994)
<i>Melandrium album</i>	Hairy root meristems	12	2	—	Veuskens <i>et al.</i> (1995), Kejnovský <i>et al.</i> (2001)
<i>Nicotiana plumbaginifolia</i>	Mesophyll protoplasts	10	0	—	Conia <i>et al.</i> (1989)
<i>Oryza sativa</i>	Root meristems	12	0	—	Lee & Arumuganathan (1999)
<i>Petunia hybrida</i>	Mesophyll protoplasts	7	1	—	Conia <i>et al.</i> (1987)
<i>Picea abies</i>	Root meristems	12	3	—	Überall <i>et al.</i> (2003)
<i>Pisum sativum</i>	Root meristems	7	2	4	Gualberti <i>et al.</i> (1996), Neumann <i>et al.</i> (2002)
	Hairy root meristems	7	2	4	Neumann <i>et al.</i> (1998)
<i>Secale cereale</i>	Root meristems	7	1	2 <sup>b</sup>	Kubaláková <i>et al.</i> (2003a)
<i>Triticum aestivum</i>	Suspension cells	21	0	—	Wang <i>et al.</i> (1992), Schwarzach <i>et al.</i> (1997)
	Root meristems	21	1(2)	2 <sup>c</sup>	Lee <i>et al.</i> (1997), Gill <i>et al.</i> (1999), Vrána <i>et al.</i> (2000), Kubaláková <i>et al.</i> (2002)
<i>Triticum durum</i>	Root meristems	14	0	2 <sup>d</sup>	Kubaláková <i>et al.</i> (2003b)
<i>Vicia faba</i>	Root meristems	6	1	6	Lucretti <i>et al.</i> (1993), Doleel & Lucretti (1995)
<i>Zea mays</i>	Root meristems	10	2(3)	—	Lee <i>et al.</i> (1996, 2002)

\*Number of chromosomes in a haploid set. \*\*Number of chromosomes that could be discriminated unambiguously. The numbers in brackets indicate the number of chromosomes that could be discriminated in some lines due to chromosome polymorphism. \*\*\*Maximum number of chromosomes discriminated in one line. Different chromosomes may be discriminated by a judicious choice of specific chromosome translocation, deletion and addition lines. <sup>a</sup>Oat–maize chromosome addition line in which maize chromosome 9 could be discriminated (Li *et al.* 2001). <sup>b</sup>Rye chromosomes 2R–7R could be discriminated from wheat–rye chromosome addition lines (Kubaláková *et al.* 2003a). <sup>c</sup>Sorting of almost all chromosome arms is possible in hexaploid wheat using individual (di)telosomic lines (Kubaláková *et al.* 2002). <sup>d</sup>All chromosome arms may be sorted from individual (di)telosomic lines (Kubaláková *et al.* 2003b).

cells, approaches based on the use of DNA synthesis inhibitors have been preferred (de Laat & Blaas 1984, Wang *et al.* 1992). The inhibitors accumulate cycling cells at the G<sub>1</sub>/S interface, and, upon release from the block, the cells transit S and G<sub>2</sub> phases and enter mitosis synchronously (Doležel *et al.* 1999a). This approach has been used to synchronize root meristems, resulting in mitotic indices of 45–62% (Doležel *et al.* 1992, Kubaláková *et al.* 2003a), with lower values for hairy root cultures (Neumann *et al.* 1998). Hydroxyurea and aphidicolin were the only two inhibitors used, the former being cheaper and far more popular. Although the use of hydroxyurea was questioned due to its potential for inducing

chromosome breakage (Eriksson 1966), de Laat & Schel (1986) were unable to detect any damage to sorted chromosomes of *H. gracilis*. This was recently confirmed by Li & Arumuganathan (2000) and Šimková *et al.* (2003) who recovered intact DNA from flow-sorted chromosomes in various species, although these studies could not exclude the presence of breaks in chromosomal DNA.

#### Accumulation of mitotic cells in metaphase

Provided the cells enter mitosis synchronously, they may be accumulated in metaphase by the action of mitotic spindle inhibitors. The alkaloid

colchicine was the first to be used (de Latt & Blaas 1984, Conia *et al.* 1987). However, as its affinity to plant tubulins is rather low, and it is effective only in millimolar concentrations, it has been replaced by synthetic herbicides such as amiprofos-methyl (Doležel *et al.* 1992), oryzalin (Veuskens *et al.* 1995) and trifluralin (Lee *et al.* 1996), which are effective at micromolar concentrations. Although the number of cells accumulated at metaphase is related to the duration of the treatment, short metaphase block has been preferred to avoid chromosomes splitting into chromatids, and to prevent chromosome decondensation (Doležel *et al.* 1992, 1994). The frequency of chromosome clumps may be further reduced by incubation in ice-cold water following treatment with the spindle inhibitor (Lysák *et al.* 1999). Optimised protocols result in accumulation of more than 50% of cells in metaphase (Lee *et al.* 1996, 1997, Vláčilová *et al.* 2002, Kubaláková *et al.* 2003a), a level considered critical for preparation of high-quality chromosome suspensions (Doležel *et al.* 1999b).

#### *Preparation of chromosome suspensions*

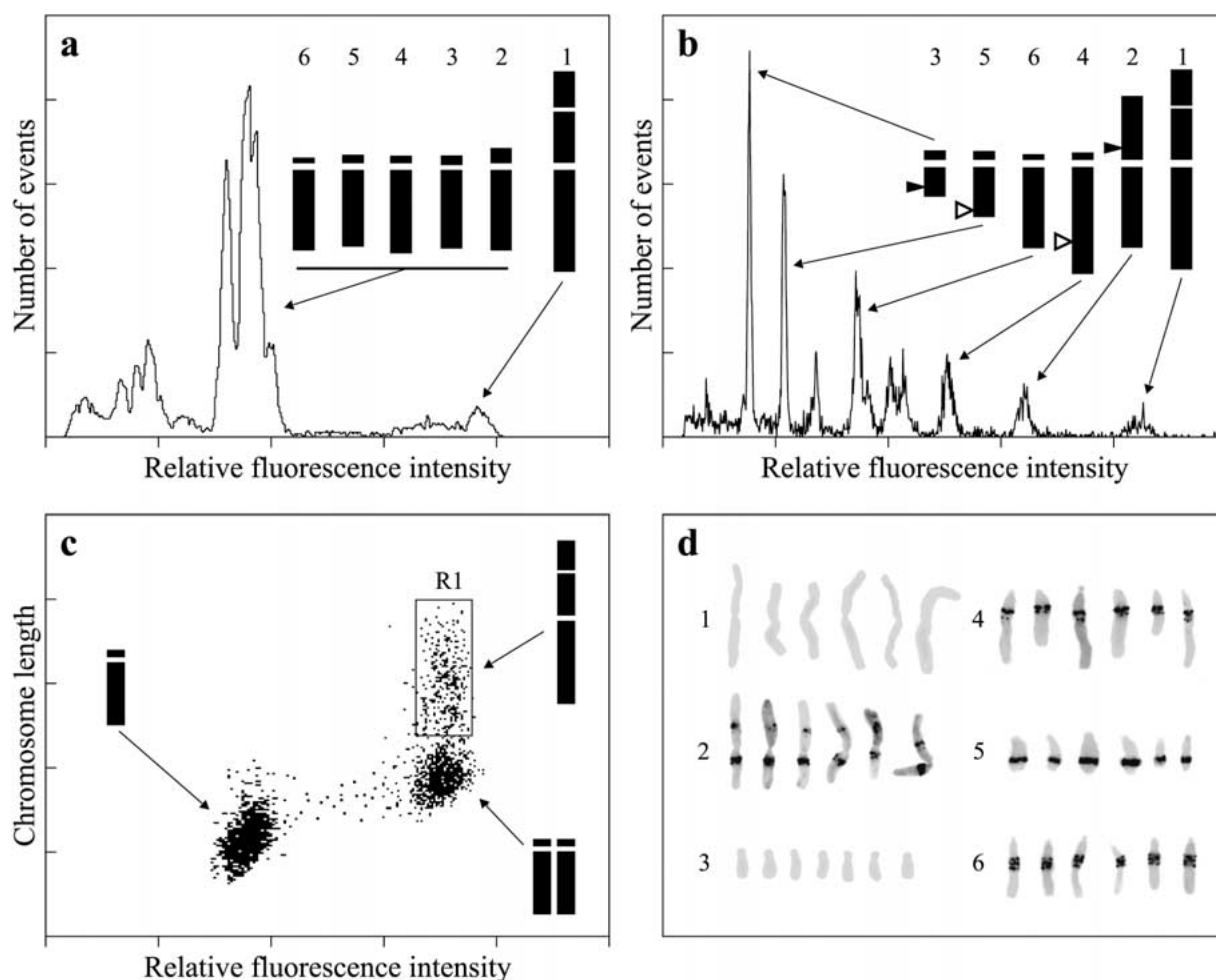
Release of chromosomes from plant cells is hampered by the presence of the rigid cell wall. Early studies used hydrolytic enzymes to remove the cell wall, and chromosomes were released after the lysis of protoplasts in a hypotonic buffer (de Laet & Blaas 1984, Arumuganathan *et al.* 1991, Wang *et al.* 1992, Veuskens *et al.* 1995). The problem with this approach was that chromosome decondensation occurred during the prolonged incubations in hydrolytic enzymes. In order to release chromosomes from root tips, Doležel *et al.* (1992) developed a novel procedure in which chromosomes are released from formaldehyde-fixed tissues by chopping with a scalpel. Subsequently, a mechanical homogenizer has been used (Gualberti *et al.* 1996). This rapid and now almost generally used method avoids long treatments with enzymes which may be contaminated by DNases. Lee *et al.* (1996) introduced a modified version of the protocol which omits the fixation step. However, the formaldehyde fixation makes chromosomes more resistant to mechanical shearing forces during flow sorting, and re-sorting

of sorted fractions is possible to achieve high purities (Lucretti *et al.* 1993). Various buffers have been used to isolate plant chromosomes (Doležel *et al.* 1994), the most frequent being the polyamine-based buffer LB01 (Doležel *et al.* 1989) and a magnesium sulphate-based buffer (Lee *et al.* 1996). Reported chromosome concentrations ranged from  $1.6 \times 10^5$  to  $1 \times 10^6$  per ml of a sample (Gill *et al.* 1999, Doležel *et al.* 1992).

#### *Flow karyotyping and discrimination of single chromosome types*

Traditionally, chromosome suspensions are stained by one or two DNA fluorochromes, and distributions of relative fluorescence intensity are displayed as monoparametric and biparametric flow karyotypes, respectively (Gray & Cram 1990). Ideally, each chromosome is discriminated as a well-separated peak. Unfortunately, this is rare in plants due to the absence of significant differences in DNA content among chromosomes, and usually only one or few chromosomes can be discriminated (Table 1, Figure 1a). The failure of bivariate flow karyotyping in plants, which relies on simultaneous staining with AT- and GC-specific dyes to facilitate chromosome discrimination on the basis of DNA composition, is most probably due to homogeneously dispersed repetitive DNA sequences (Lucretti & Doležel 1997, Lee *et al.* 1997, 2000). Despite some promising results with the labelling of specific repetitive sequences on chromosomes in suspension (Pich *et al.* 1995, Macas *et al.* 1995), this approach has never become routine as it is hard to achieve quantitative labelling (Doležel *et al.* unpublished).

The recent progress in plant flow cytogenetics has been stimulated by a judicious use of cytogenetic stocks. Lucretti *et al.* (1993) and Doležel & Lucretti (1995) showed that field bean chromosomes whose length has been altered by translocation could easily be discriminated (Figure 1b). Since then, a whole range of cytogenetic stocks, including deletions, translocation and chromosome additions, has been found useful to discriminate specific chromosomes and chromosome arms in a variety of species (Table 1 Gill *et al.* 1999, Li *et al.* 2001, Kubaláková *et al.* 2002, 2003a, 2003b). Simulated flow karyotypes have been employed to



**Figure 1.** Chromosome analysis and sorting in field bean (*Vicia faba*,  $2n=12$ ). **(a)** Flow karyotype of cv. Inovec with wild-type chromosomes. Five acrocentric chromosomes (2–6) have similar size, and form a composite peak. Only the largest metacentric chromosome 1 can be discriminated and sorted. **(b)** Flow karyotype of a translocation line EF, whose chromosomes differ in size due to two reciprocal translocations (breakpoints marked by arrowheads). All six chromosomes are discriminated as well-separated peaks and may be sorted. Non-labelled peaks represent chromatids and chromosome arms. **(c)** Dot-plot of fluorescence intensity versus chromosome length obtained in cv. Inovec. Fluorescence intensity of doublets of acrocentric chromosomes is similar to that of the large metacentric chromosome 1, and the chromosome may be sorted at high purity by choosing a sort window (R1), which avoids the doublets (Lucretti *et al.* 1993). **(d)** Examples of chromosomes sorted from line EF; note the variation in length. The chromosomes can be identified unambiguously after fluorescent labelling of the *FokI* repeat using PRINS (Doležel *et al.* 1999b). The colour image was converted to grey scale.

predict a feasibility of discriminating individual chromosome types depending on differences in relative DNA content and on the coefficient of variation of the chromosome peaks (Lysák *et al.* 1999, Lee *et al.* 2002). The karyotypes were modelled using computer spreadsheet software (Conia *et al.* 1989) or a dedicated computer program (Doležel 1991) based on relative chromosome length or relative chromosomal DNA content.

#### Chromosome sorting

Any chromosome or group of chromosomes that can be discriminated on a flow karyotype can be sorted (Figure 1a, b, d). Plant chromosomes have been sorted onto nylon filters, microscope slides, and into PCR and other tubes (de Laat & Blass 1984, Conia *et al.* 1987, Arumuganthan *et al.* 1994). Compared with humans and animals, plant

chromosome suspensions are less concentrated and the sorting speed with conventional flow-sorters ranged from 5 to 80 chromosomes/s (de Laat & Blaas 1984, Lysák *et al.* 1999). Although the sorting rate might be increased using high-speed sorters, a low sample density will remain a limiting factor. During a large-scale chromosome-sorting experiment, Šafář *et al.* (2003) were able to sort  $2 \times 10^5$  wheat chromosomes during one working day. Sorting of millions of chromosomes is therefore possible but represents a major exercise. On the other hand, sorting of hundreds or thousands of chromosomes is easy.

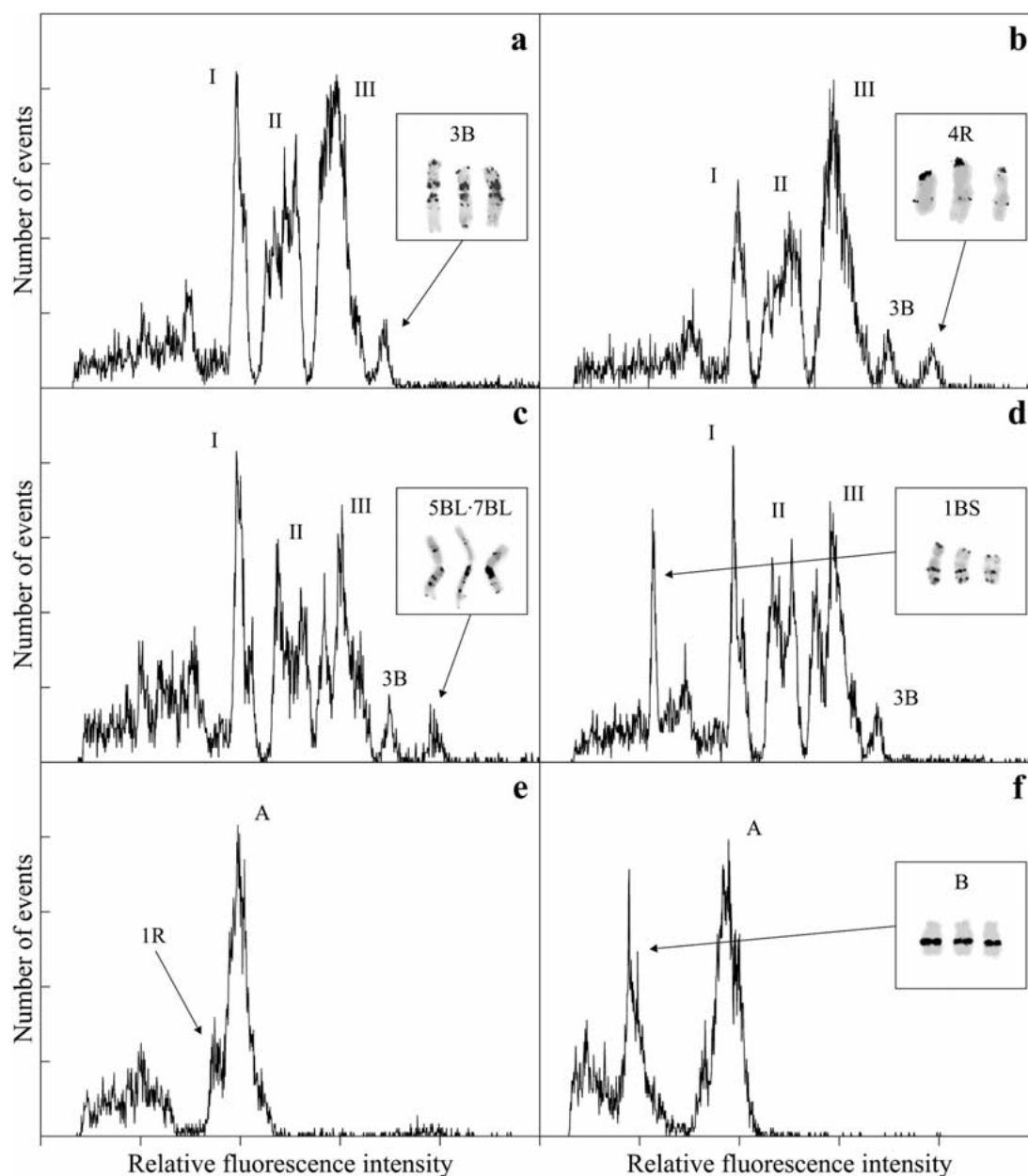
The usefulness of flow-sorted fractions depends on the extent of contamination with other chromosomes and their parts, which may be determined microscopically (de Laat & Blass 1984). However, as the chromosome morphology may be changed after sorting, molecular cytogenetic techniques including fluorescence *in-situ* hybridization (FISH) and primed *in-situ* DNA labelling (PRINS) have been preferred (Kubaláková *et al.* 2000, Li *et al.* 2001; Kubaláková 2003a, Figure 1d). Dot-blotting and, more frequently, PCR with specific primers have also been used (Arumuganathan *et al.* 1994, Lysák *et al.* 1999, Vrána *et al.* 2000) but are not suitable for determining the frequency of individual contaminants (Kubaláková *et al.* 2000). In some cases, the purity in sorted chromosome fractions has reached 100% (Lysák *et al.* 1999, Vrána *et al.* 2000, Vláčilová *et al.* 2002). The purity was found to depend on the degree of resolution of individual chromosomes, and the presence of particles and clusters of particles with the same DNA content as the sorted chromosome (Doležel *et al.* 2001). The purity may be improved by appropriate gating to select a subpopulation of particles for further signal processing, and by properly setting a sort window which defines the sorted population. Conia *et al.* (1987) found that discrimination of *Petunia* chromosomes could be improved by simultaneous analysis of forward-angle light scatter and relative DNA content. Lucretti *et al.* (1993) showed that a sort window selecting particles based on relative fluorescence intensity and length improved the purity in a sorted fraction of field bean chromosomes (Figure 1c). They also demonstrated that two-stage sorting reduced contamination with doublets of chromosomes whose length is similar, as the doublets were dissociated during the first sort run.

## Applications

### *Flow karyotyping*

Doležel *et al.* (1994) reasoned that flow karyotyping should be sensitive enough to detect numerical and structural chromosome changes in plants, including chromosome polymorphism. During the last decade, these predictions have been fulfilled. The ability to detect numerical chromosome changes was demonstrated in barley, where trisomy of chromosome 6 could be identified easily (Lee *et al.* 2000). The presence of an alien chromosome was detected in an oat–maize addition line (Li *et al.* 2001) and in six different wheat–rye addition lines, where flow karyotyping also permitted monitoring the frequency of alien chromosomes in the population (Kubaláková *et al.* 2003a; Figure 2a, b). The ability to recognise chromosome translocations and deletions has been documented in field bean, garden pea, barley, rye and wheat (Doležel & Lucretti 1995, Neumann *et al.* 1998, Gill *et al.* 1999, Lysák *et al.* 1999, Vrána *et al.* 2000, Kubaláková *et al.* 2002, 2003a; Figure 2c, d). By flow karyotyping, Kubaláková *et al.* (2002) discovered a translocation chromosome 5BL·7BL in seven wheat cultivars where its presence was not known before (Figure 2c). In wheat cultivars ‘Panthus’ and ‘Sida’ they identified a chromosome 4D, whose length had increased due to an unknown duplication or translocation. Finally, Kubaláková *et al.* (2003a) discovered B chromosomes in a rye cultivar ‘Adams’ (Figure 2e, f).

Generally, chromosomes are only detected if their size has been changed so that they differ enough from the remaining chromosomes. However, the case of the 1BL·1RS translocation in wheat shows that even a poorly discriminated chromosome may be detected by a characteristic change in the flow karyotype (Kubaláková *et al.* 2002). Chromosome deletions have also been identified in wheat using flow karyotyping (Gill *et al.* 1999, Kubaláková *et al.* 2002). Furthermore, flow karyotyping was found to be sensitive enough to detect polymorphism in relative DNA content of chromosomes in wheat, rye, maize and barley, and the ‘fingerprint’ patterns of flow karyotypes characteristic for certain cultivars are heritable (Lee *et al.* 2000, 2002, Kubaláková *et al.* 2002,



**Figure 2.** Chromosome analysis and sorting in cereals. **(a)** Flow karyotype of wheat (*Triticum aestivum*,  $2n=6x=42$ ) cv. Chinese Spring. Only chromosome 3B can be discriminated and sorted. Remaining chromosomes form three composite peaks (I–III). **(b)** Identification of a rye chromosome 4R on a flow karyotype of a wheat–rye chromosome addition line. **(c)** Discovery of a translocation chromosome 5BL·7BL in wheat cv. Famulus. **(d)** Flow karyotype of a ditelosomic line of wheat showing a well-discriminated peak containing the short arm of chromosome 1B (1BS). **(e)** Flow karyotype of rye (*Secale cereale*,  $2n=14$ ) cv. Selgo. All seven chromosomes form a composite peak (A) with a shoulder containing chromosome 1R. No chromosome can be sorted at high purity from this variety. **(f)** Discovery of supernumerary B chromosomes in rye cv. Adams. Inserts: examples of sorted chromosomes after fluorescent labelling of GAA microsatellites **(a, c, d)** using PRINS (Kubaláková *et al.* 2002), and repeated sequences pSc119 **(b)** and *Afa* **(f)** using FISH (Kubaláková *et al.* 2003a). Colour images were converted to grey scale.

2003a). It should be noted that the currently used protocols require a large number of root tips to prepare chromosome suspensions. However, Gualberti *et al.* (1996) demonstrated that a chromosome suspension could be prepared from a single root, demonstrating the possibility of analysing chromosomes of individual plants.

#### *Chromosome sorting*

Although chromosomal DNA is the obvious target, isolated plant chromosomes were found to be suitable for ultrastructural analysis using scanning electron microscopy, and for protein analysis. Compared with other protocols (Wanner *et al.* 1991), large numbers of chromosomes may be prepared, they are not covered by remnants of cytoplasm, and their morphology is well maintained (Schubert *et al.* 1993). The same authors showed that, for the same reasons, isolated chromosomes were an ideal target for immunolocalization of proteins. This opportunity was explored by Binarová *et al.* (1998) to demonstrate  $\gamma$ -tubulin in the kinetochore/centromeric regions of plant mitotic chromosomes. Subsequently, ten Hoopen *et al.* (2000) used flow-sorted field bean and barley chromosomes in a detailed study on plant kinetochore proteins.

Lucretti *et al.* (1993) and Gualberti *et al.* (1996) showed that chromosomes sorted onto microscope slides were suitable for localization of DNA sequences using FISH and PRINS, respectively. In these studies, and in many others that followed, FISH and PRINS were used to identify flow-sorted chromosomes (Lysák *et al.* 1999, Vrána *et al.* 2000, Neumann *et al.* 2002, Vláčilová *et al.* 2002, Kubaláková *et al.* 2003a, 2003b). Compared with conventionally prepared metaphase spreads, sorted chromosomes appear to be an attractive target for FISH and PRINS, as they are completely free of cytoplasmic contamination and thus facilitate high-resolution physical cytogenetic mapping. As thousands of chromosomes may be analysed on one slide, screening of large chromosome populations is feasible. For example, the analysis of one thousand sorted chromosomes is equivalent to screening five hundred metaphase plates. This approach facilitated the analysis of the intravarietal polymorphism in genomic distribution of GAA clusters in wheat

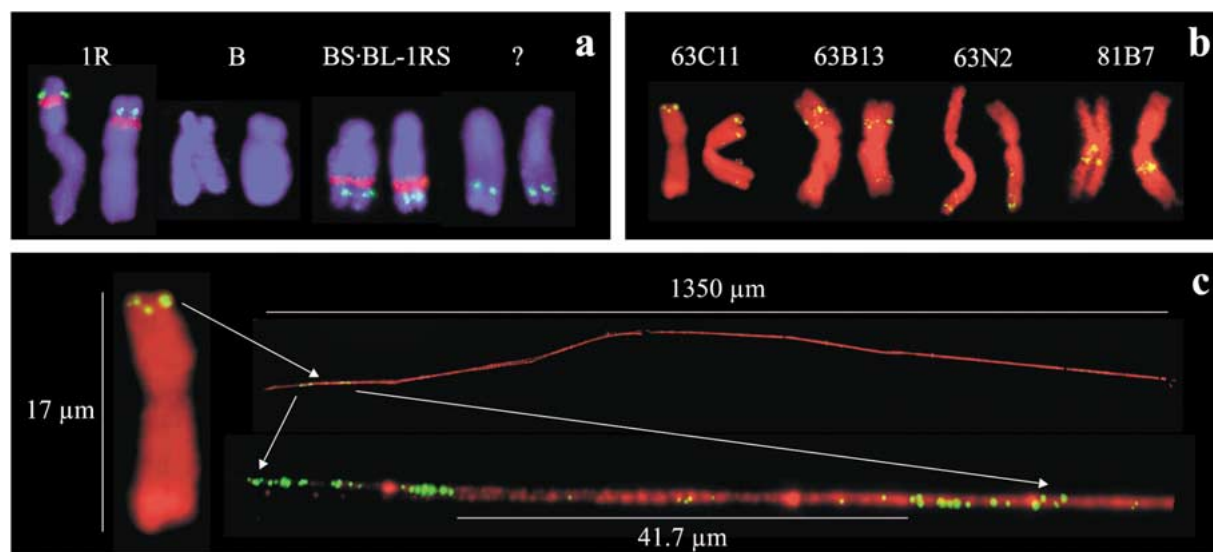
(Kubaláková *et al.* 2002), and the identification of a rare translocation between A and B chromosomes in rye (Kubaláková *et al.* 2003a; Figure 3a). The power of FISH on flow-sorted chromosomes has been greatly increased since a protocol for preparation of super-stretched chromosomes was developed (Valárik *et al.* 2003). Sorted chromosomes may be elongated up to a hundred fold compared with untreated chromosomes, making them outstanding material for high-resolution physical mapping (Figure 3c). The technique is especially attractive for plant species with large genomes as an alternative to FISH on pachytene chromosomes, which are generally too long to trace individually (de Jong JH *et al.* 1999).

The most frequent use of flow-sorted human and animal chromosomes has been the preparation of chromosome painting probes from chromosome libraries (Cremer *et al.* 1988, Pinkel *et al.* 1988) or from small numbers of sorted chromosomes after PCR amplification and DNA labelling (Telenius *et al.* 1992). Despite great efforts, attempts to paint plant chromosomes using probes prepared from flow-sorted chromosomes have failed. Presumably, because of the presence of large amounts of homogeneously dispersed repetitive DNA sequences in plant genomes, the painting probes prepared so far hybridized to all chromosomes within a set (Schubert *et al.* 2003).

An attractive application of flow-sorted chromosomes is physical mapping of DNA sequences using dot blotting and PCR. In order to assign individual chromosomes to peaks on the flow karyotype, Arumuganathan *et al.* (1994) sorted tomato chromosomes from well-resolved peaks onto membrane filters and used them for hybridization with chromosome-specific repetitive DNA probes. As this method requires ten thousand chromosomes to be sorted for each spot, which is time-consuming, it has never been used for mapping. On the other hand, only hundreds of chromosomes are sufficient for PCR with sequence-specific primers. As sorting of hundreds of chromosomes takes only a few minutes, large screening panels may be prepared for high-throughput mapping using PCR.

Macas *et al.* (1993) used PCR on sorted chromosomes to localize seed storage protein genes in field bean. In order to overcome difficulties of discriminating single chromosome types, they sorted





**Figure 3.** Physical mapping of DNA sequences on flow-sorted chromosomes using FISH. DAPI counterstain is shown in blue (a) and red pseudocolour (b, c). (a) Discovery of two rare translocations between A and B chromosomes in rye (*Secale cereale*). Flow-sorted rye chromosomes 1R, B chromosomes, and two translocation chromosomes were used for FISH with probes for 45S rDNA (red colour) and 5S rDNA (yellow colour). Two examples are shown for each chromosome and demonstrate the presence of the rDNA loci on 1R and their absence on the B chromosome. A translocation between a B chromosome and short arm of a rye chromosome 1R (BS·BL-1RS) could be identified based on the presence of both 45S and 5S rDNA. Using the rDNA probes, it was not possible to decide whether 5S rDNA bearing chromosome 1R or 5R was involved in a second translocation (marked by ?) (Kubaláková *et al.* 2003a). (b) Localization of BAC clones on flow-sorted chromosome 3B of wheat (*Triticum aestivum*) using FISH; the four BAC clones (63C11, 63B13, 63N2, and 81B7) provide specific chromosome landmarks (yellow signals). For each probe, two representative examples are given (Šafář *et al.* 2003). (c) The use of super-stretched chromosomes for high-resolution cytogenetic mapping. FISH with a wheat BAC clone 63C11 detects two sites on each chromatid separated by 0.2 µm on the untreated chromosome (17 µm long). The distance increases to 41.7 µm on a chromosome that has been stretched to 1350 µm, resulting in significant improvement in spatial resolution of the region of interest (Valárik *et al.* 2003).

chromosomes from translocation lines. This permitted gene mapping at the subchromosomal level and integration of genetic and physical maps (Macas *et al.* 1993, Vaz Patto *et al.* 1999). Lysák *et al.* (1999) used the same approach to localize two RFLP probes to regions of two chromosomes of barley. PCR on flow-sorted chromosomes was also used by Kejnovský *et al.* (2001) to localize male-specifically-expressed *MROS* genes on sex chromosomes and autosomes of a dioecious plant, white campion (*Silene latifolia*), and by Vláčilová *et al.* (2002), who were the first to assign a genetic linkage group to a specific chromosome in chickpea. Although the garden pea has been the object of genetic studies since Gregor Mendel, the assignment of two genetic linkage groups to specific chromosomes remained unclear until Neumann *et al.* (2002) used flow-sorted pea chromosomes for PCR detection of

molecular markers selected from these unassigned groups. This elegant work marked the completion of efforts to integrate genetic and physical maps of the pea (Figure 4).

Flow-sorted chromosomes have been used not only for physical mapping of molecular markers but also for the development of markers for mapping. This approach is especially attractive for saturating genetic maps in the regions harbouring agronomically important traits. Arumuganathan *et al.* (1994) developed eleven RFLP markers from tomato chromosome 2. Macas *et al.* (1996) and Koblížková *et al.* (1998) showed that libraries constructed from flow-sorted chromosomes contain microsatellite sequences, and that the libraries can be efficiently enriched for these loci to facilitate their screening for microsatellite markers. More recently, Požárková *et al.* (2002) developed microsatellite markers from chromosome 1 of

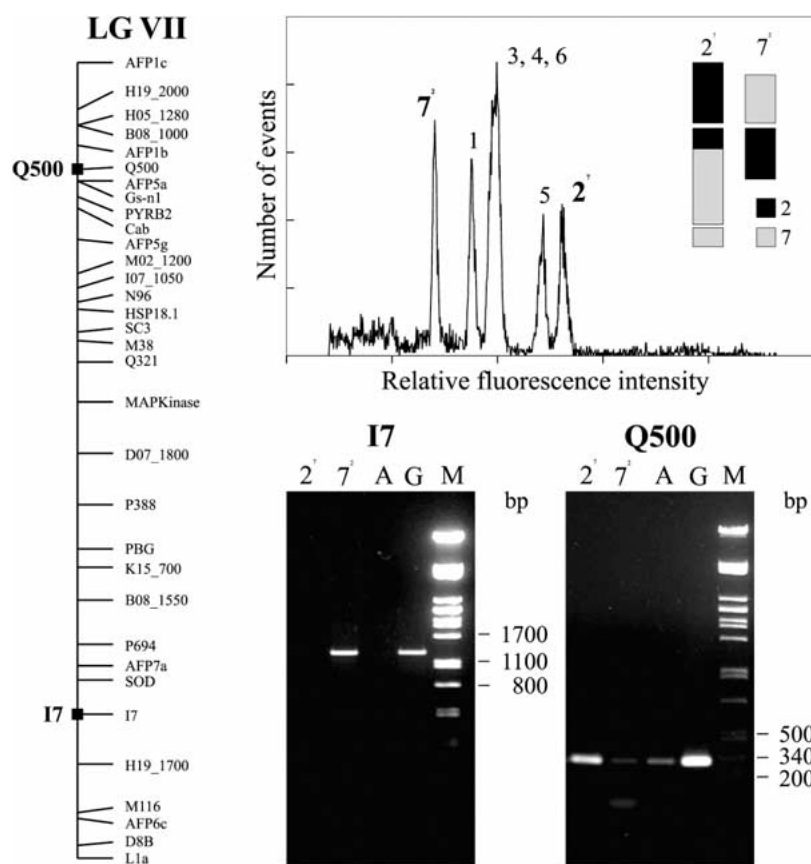


Figure 4. Physical mapping and assignment of a genetic linkage group to a specific chromosome in garden pea (*Pisum sativum*,  $2n = 14$ ). Molecular markers I7 and Q500 were selected from linkage group LG VII, which was thought to be located on either chromosome 4 or 7. The linkage group was unquestionably mapped to chromosome 7 using PCR on 300 translocation chromosomes  $2^7$ ,  $7^2$ , and on 1500 chromosomes of the remaining types (lanes A) flow-sorted from a translocation line JI148. PCR products were resolved using agarose gel electrophoresis. Lanes G: total genomic DNA; lanes M: lambda DNA digested with *Pst*I used as a marker. Note that the use of translocation chromosomes facilitated mapping of markers to subchromosomal regions (Neumann *et al.* 2002, modified).

field bean. This chromosome is the largest in the karyotype and trisomic lines, which have been used for genetic mapping in this crop (Vaz Patto *et al.* 1999), are not available for chromosome 1. In their work, Požárková *et al.* (2002) used flow-sorted fractions not only to develop the markers but also to confirm their chromosome specificity, thus highlighting the effectiveness of the experimental system. Both Arumuganathan *et al.* (1994) and Požárková *et al.* (2002) selected markers from short-insert chromosome-specific DNA libraries.

The utility of DNA libraries depends on the size of inserts, genome coverage, and contamination with unwanted sequences. In contrast to

whole genome libraries, chromosome-specific libraries represent defined genome fractions and their use simplifies the analysis of large genomes. The first DNA library prepared using flow-sorted plant chromosomes was prepared by Wang *et al.* (1992). Although the identity of sorted wheat chromosomes could not be verified, half of the unique and low-copy clones selected from the library detected sequences on wheat chromosome 4A. To overcome the need for sorting large numbers of chromosomes required for direct cloning, Arumuganathan *et al.* (1994) and Požárková *et al.* (2002) employed DOP-PCR amplification to increase the amount of DNA prior to cloning. As a result of this, the libraries

had insert sizes in the range of  $10^2$ – $10^3$  bp. Following the same approach, Macas *et al.* (1996) were the first to prepare a complete set of chromosome-specific libraries covering the whole genome of the field bean. So far, the field bean is the only plant species whose genome is available in the form of chromosome-specific DNA libraries.

Although the short insert DNA libraries are useful tools, libraries with large ( $\sim 10^5$  bp) inserts (e.g. cloned in a BAC vector) are more important as they facilitate construction of physical maps and gene cloning, and are a source of material for other studies and techniques, such as BAC FISH (Figure 3b). Their construction from sorted chromosomes has been hampered by a need for large quantities of high-molecular-weight DNA. In 2000, two groups reported recovery of high-molecular-weight DNA from plant chromosomes. Li & Arumuganathan (2000) obtained DNA over 120 kb in size from sorted maize chromosomes, and Vrána *et al.* (2000) showed that majority of DNA obtained from flow-sorted wheat chromosomes was megabase sized (Figure 5). Subsequently, Šimková *et al.* (2003) developed a protocol for preparation of high-molecular-weight DNA from sorted plant chromosomes. DNA thus obtained was fully digestible by restriction enzymes and suitable for cloning. This progress facilitated construction of a subgenomic BAC library from wheat chromosomes 1D, 4D and 6D (Janda *et al.* in preparation) and the first ever chromosome-specific BAC library from wheat chromosome 3B (Šafář *et al.* 2003). The most recent results from Janda *et al.* (in preparation) demonstrate the possibility of preparing a BAC library specific for a chromosome arm after sorting the wheat telocentric chromosome 1BS from a ditelosomic line.

#### Limitations and comparison with other methods

In addition to flow cytometry, microdissection has been used to isolate plant chromosomes. Each method has advantages and disadvantages and both appear to be surprisingly compatible. While any chromosome or chromosome segment may be isolated using microdissection at 100%

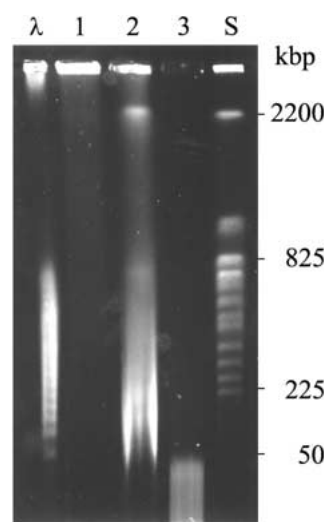


Figure 5. Analysis of the quality of the DNA obtained from flow-sorted chromosomes of wheat (*Triticum aestivum*). Agarose plugs containing chromosomal DNA were incubated in *Hind*III buffer containing 0 (lane 1), 5 (lane 2) or 25 units (lane 3) of *Hind*III restriction enzyme for 5 min (lanes 1 and 2) and 2 h (lane 3) at 37°C and analysed using pulse field gel electrophoresis. Untreated DNA remained in the well (lane 1) suggesting that DNA of sorted chromosomes was intact and of megabase size. At the same time, the DNA was easily accessible to the restriction endonuclease (lanes 2, 3). Lane  $\lambda$ : lambda ladder; lane S: chromosomes of *Saccharomyces cerevisiae* used as size markers (Vrána *et al.* 2000, modified).

purity, flow sorting relies on intrinsic differences in chromosome size and on the use of specific stocks; the purity is usually less than 100%. Only a very small number of chromosomes may be isolated by microdissection and any work requiring larger amounts of DNA must involve PCR amplification, which results in a collection of short DNA fragments, and which may introduce a bias (Telenius *et al.* 1992, Macas *et al.* 1996). Flow cytometry purifies large numbers of chromosomes and high-molecular-weight DNA may be obtained. The possibility of sorting large numbers of chromosomes allows the preparation of many fractions for high-throughput analysis using PCR, and for microscopic analysis. Unlike microdissected chromosomes, flow-sorted chromosomes are suitable for both protein immunolocalization and isolation. Although the currently used protocols for chromosome isolation may easily be modified for other species, their optimization is laborious and time-consuming. One

may therefore envisage flow cytogenetics being used with a limited number of species of important scientific (e.g., evolution of sex chromosomes) or economic (e.g., gene mapping and isolation) interest. A need for special stocks to discriminate specific chromosomes will also limit the number of species where flow cytogenetics could be used.

### Future directions

Chromosome-mediated gene transfer was probably the first motivation for chromosome sorting in plants (de Laat & Blaas 1984). Although it is very attractive for both the transfer and mapping of polygenic traits, this goal has not yet been attained. Flow sorting may also aid in preparation of artificial plant chromosomes in significant quantities, as has been shown in humans (de Jong *G et al.* 1999). The use of large numbers of mitotic chromosomes for proteomic characterization of chromatin is another avenue to explore. Due to the use of highly purified chromosome fractions, this high-throughput approach has the potential for identifying less abundant and hitherto unknown proteins specific to mitotic chromosomes.

The applications of sorted chromosomes for targeted isolation of molecular markers and for rapid physical mapping using PCR are especially attractive for analysis of complex genomes, which are typical of major legume and cereal crops. As the methods are available for many of these species (Table 1), one may foresee their large-scale use. In addition to PCR screening, hybridization to DNA arrays offers a route to high-throughput physical mapping of various DNA sequences, including expressed sequence tags (ESTs). As huge numbers of ESTs have been generated (Mayer & Mewes 2002), this may be an attractive approach to facilitating their assignment to individual chromosomes and their parts. HAPPY mapping is an *in-vitro* technique, which defines the order and spacing of DNA markers directly on native genomic DNA. It appears to be an attractive alternative to *in-vivo* genetic and physical mapping and is suitable for preparation of maps of entire plant genomes up to 1000 Mb (Waugh *et al.* 2002). As the genomes of most crops are much larger, purification of individual chromo-

somes and chromosome arms may greatly expand the usefulness of HAPPY mapping in plants.

Dissection of a genome into single chromosomes and arms should facilitate analysis of sequence organization and evolution of repetitive DNA sequences (such as telomeric and subtelomeric tandem repeats) at the chromosomal and subchromosomal levels. As thousands of chromosomes may be analysed microscopically on a single slide, there are several potential applications of sorted chromosomes in the area of molecular cytogenetics. These could include high-resolution mapping of DNA sequences, analysis of polymorphism of DNA repeats, and discovery of rare structural chromosome changes. The application of super-stretched chromosomes may fill the gap in spatial resolution and sensitivity between FISH on pachytene chromosomes and FISH on DNA fibres. Preparation of chromosome painting probes directly from sorted plant chromosomes seems unlikely. However, if a sufficient number of ESTs is collected, e.g., by hybridization of labelled DNA from sorted chromosomes to DNA arrays, selected ESTs could be pooled to prepare complex chromosome-specific probes.

The recent success in the creation of chromosome- and chromosome-arm-specific BAC libraries represents a great leap forward, and marks the integration of plant flow cytogenetics with genomics. Although genomic BAC libraries exist for most of the important crops, their maintenance and screening may be laborious and costly. A BAC library containing 5.1 genome equivalents of tetraploid wheat consists of 516096 clones (Cenci *et al.* 2003). In contrast, the BAC library specific for a single wheat chromosome, 3B, with a higher genome coverage ( $6.2 \times$ ) consists of only 67968 clones (Šafař *et al.* 2003). Dissection of complex genomes into chromosome- and chromosome-arm-specific BAC libraries is now feasible and should greatly facilitate the study of genome organization, map-based cloning, physical mapping and genome sequencing.

### Acknowledgements

We are grateful to Ms. Radka Tušková and Jitka Weiserová, Bc. for excellent technical assistance.

This work was supported by research grants 521/03/0595 and 522/03/0354 from the Grant Agency of the Czech Republic, QC1336 from the Ministry of Agriculture of the Czech Republic, and ME527 and ME528 from the Ministry of Education, Youth and Sports of the Czech Republic.

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