

Chromosome analysis and sorting

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Abstract

Flow cytometric analysis and sorting of plant mitotic chromosomes has been mastered by only a few laboratories worldwide. Yet, it has been contributing significantly to progress in plant genetics, including the production of genome assemblies and the cloning of important genes. The dissection of complex genomes by flow sorting into the individual chromosomes that represent small parts of the genome reduces DNA sample complexity and streamlines projects relying on molecular and genomic techniques. Whereas flow cytometric analysis, that is, chromosome classification according to fluorescence and light scatter properties, is an integral part of any chromosome sorting project, it has rarely been used on its own due to lower resolution and sensitivity as compared to other cytogenetic methods. To perform chromosome analysis and sorting, commercially available electrostatic droplet sorters are suitable. However, in order to resolve and purify chromosomes of interest the instrument must offer high resolution of optical signals as well as stability during long runs. The challenge is thus not the instrumentation, but the adequate sample preparation. The sample must be a suspension of intact mitotic metaphase chromosomes and the protocol, which includes the induction of cell cycle synchrony, accumulation of dividing cells at metaphase, and release of undamaged chromosomes, is time consuming and laborious and needs to be performed very carefully. Moreover, in addition to fluorescent staining chromosomal DNA, the protocol may include specific labelling of DNA repeats to facilitate discrimination of particular chromosomes. This review introduces the applications of chromosome sorting in plants, and discusses in detail sample preparation, chromosome analysis and sorting to achieve the highest purity in flow-sorted fractions, and their suitability for downstream applications.

KEYWORDS

cell cycle synchronization, mitotic metaphase chromosomes, liquid chromosome suspension, DNA isolation, DNA amplification, gene mapping and cloning, genome sequencing, marker development, repetitive DNA labelling

1 | INTRODUCTION

Studies initiated during the last quarter of the nineteenth century gradually revealed the key roles of chromosomes in storing and

transmitting hereditary information and in generating genetic variation. The most frequent method to study chromosome organization and behavior had been optical microscopy. However, in the last quarter of the twentieth century, efforts were made to employ flow

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cytometry for fast and quantitative characterization of the human chromosome complement (flow karyotyping) to replace laborious microscopic observation [1, 2]. Unfortunately, these expectations were not fulfilled. Instead, molecular cytogenetics was established and provided much higher resolution and more detailed information concerning the molecular organization of human, animal and plant chromosomes. Although flow karyotyping cannot compete with molecular cytogenetics, it is fair to point to a few exceptions, such as in the analysis of wheat cultivars where it detected recombinant chromosomes whose presence was previously unknown [3].

The availability of methods for flow cytometric chromosome analysis also opened avenues for purification of specific chromosomes by flow sorting. Interestingly, this method contributed to the development of chromosome painting [4, 5] that revolutionized mammalian cytogenetics. Chromosome sorting also played an important role at the beginning of the human genome sequencing project through facilitating the mapping of genes to chromosomes and the development of chromosome-specific DNA libraries [6, 7]. In plants, chromosome sorting has been employed in many applications, ranging from the targeted development of DNA markers [8–11], the construction of DNA libraries [12, 13], gene cloning [14, 15], genome sequencing [16–18], and the validation of whole genome shotgun sequence assemblies [19, 20].

As compared to dealing with the whole genome, chromosome sorting offers a massive and lossless reduction of DNA sample complexity, and this both simplifies the analysis of DNA sequence data and reduces project costs. However, there is an additional group of applications where sorting all chromosomes of the chromosome complement is beneficial. Since the purified sample consists only of mitotic chromosomes, this provides the opportunity to characterize the proteome of mitotic metaphase chromosomes and to reveal spatial organization of chromosomal DNA using the chromosome conformation capture methods. In the following, we describe chromosome analysis and sorting in higher plants, pointing to critical steps and outlining the methods for selected applications.

2 | SAMPLE PREPARATION

2.1 | Choice of plant material

A majority of cells forming a plant organism are in interphase. Cells in the metaphase stage of the cell cycle, which is the only stage at which condensed chromosomes are physically separated from each other, are rare or completely absent. In order to increase their frequency and accumulate cells at metaphase, cells must be induced to cycle and divide. In principle, different plant parts, organs, and cell suspensions may be used, and a number of attempts have been made to verify their suitability. Suspension cultured cells [21–23] initially seemed an ideal system, since cells grown *in vitro* under controlled conditions may be easily treated with chemical agents in order to induce mitotic synchrony. However, cell cultures cannot be easily established in every species [24], and they are often

genetically heterogeneous [22] and karyologically unstable [25]. A majority of cells in differentiated tissues *in vivo*, such as leaf mesophyll are arrested at G₁ phase of cell cycle. As all cells are initially at the same cell cycle stage, they should traverse S and M phases synchronously upon cell cycle induction. Unfortunately, the experiments by Conia et al. [26] did not confirm this expectation and mitotic synchrony observed after transferring isolated mesophyll protoplasts to a nutrient medium was low.

Buds and root tips belong to a few plant organs comprising proliferating cell populations, with root tips having large populations of cycling cells that are easy to manipulate [27, 28]. Seeds are available for many plant species, and young seedlings can be grown hydroponically and their roots treated with chemical agents to achieve high proportions of metaphase cells [27, 29]. An important advantage is that the meristem cells are karyologically stable. If a certain genotype can only be propagated vegetatively, actively-growing roots may be obtained from genetically transformed “hairy” root cultures [30, 31]. It is also possible to grow vegetative organs, such as bulbs or stem cuttings, using hydroponic systems to initiate root formation and growth [32, 33]. Due to these advantages, almost all experiments on chromosome analysis and sorting in plants reported so far have utilized samples prepared from synchronized root tip meristems [34–36]. The subsequent parts of this paper will deal with this type of source tissue.

2.2 | Cell cycle synchronization and accumulation of cells in metaphase

In order to enable treatments with various compounds to achieve high frequency of cells at metaphase, it is recommended to grow seedlings in a hydroponic system (Figure 1). The nutrient medium should have a defined composition and Hoagland's solution [37] has been generally used [27]. Its concentration should be adjusted so that it does not negatively affect mitotic activity in root tip meristems. As cell cycle

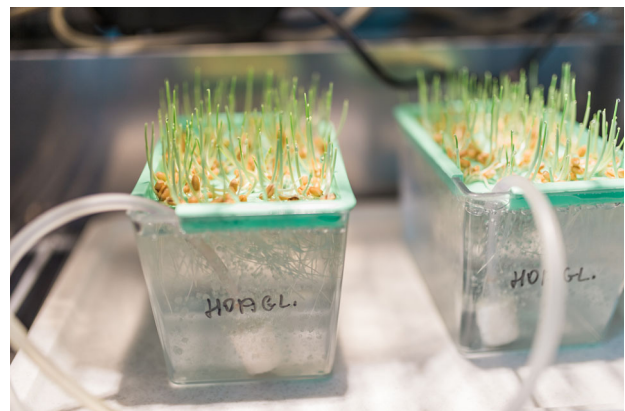


FIGURE 1 Plastic trays with open-mesh baskets supporting young seedlings grown in hydroponic. Note that the Hoagland's nutrient solution is aerated using aquarium bubble air stones to avoid hypoxia. Photo Pavlína Jáchimová, ASCR

kinetics is sensitive to temperature, all treatments in such system are performed at constant temperature in a biological incubator, the solutions are pre-heated to the required temperature, and are also aerated to ensure an adequate oxygen supply.

Root tips of young seedlings are first treated with a DNA synthesis inhibitor to accumulate a majority of cycling population at G1-S interface. Hydroxyurea, a compound which inhibits ribonucleotide reductase [38], has been by far the most frequently used because of its effectiveness and low cost. After the cycling cells are accumulated at the G1-S interface, the seedlings are transferred to inhibitor-free nutrient medium to allow the cells to resume the cell cycle and to traverse synchronously through S and G2 phases. Cytological evaluation of the proportion of mitotic cells at different time points following the release from the block, helps to identify the recovery time at which the highest proportion of cells reaches mitosis. In addition, flow cytometric analysis of DNA content of meristem tip nuclei makes it possible to follow the progression through the cell cycle of the synchronized population after the release of the block (Figure 2). The results identify the optimal concentration of DNA synthesis inhibitor and the treatment duration to accumulate a majority of cells at the G1-S interface and yet allow a rapid transit through S and G2 after the release from the block [27]. The treatments optimized for a range of plant species are listed in Table 1. In fact, it has been found that the highest degree of mitotic synchrony is achieved at hydroxyurea concentrations that allow the cells arrested at G1-S interface

to escape the block a few hours before the roots are transferred to hydroxyurea-free solution (Figure 2) [27].

In order to accumulate mitotic cells at metaphase, seedlings are transferred to a medium supplemented with mitotic spindle inhibitor. From a range of compounds with this property, only a few synthetic herbicides have been used, the most popular being amiprofos-methyl, followed by oryzalin and trifluralin [29, 55, 56]. Their advantage over the traditionally used colchicine is that they are effective in lower concentrations, are less toxic to plant cells and are easier to manipulate. It is recommended that the compounds be used at the lowest concentration that arrests chromosomes at metaphase and that the treatment time is kept as short as possible (e.g., limited to about 2 h) to avoid separation of sister chromatids (Table 1). The optimum timing of the metaphase block to achieve the highest frequency of cells at metaphase is determined cytologically. In some species, including maize and rapeseed, the chemicals inhibiting mitotic spindle may induce chromosome clumping. In maize, this can be avoided by replacing common spindle inhibitors by a treatment with nitrous oxide [57]. In some species, spreading of metaphase chromosomes across the cell volume can be improved by an ice-water treatment [33]. Seedlings with synchronized roots may be treated with ice-water overnight, allowing convenient scheduling of the next step of the protocol for the following morning. If the overnight treatment is not part of the protocol, root tips must be fixed immediately after the metaphase block to avoid separation of sister chromatids and chromosome decondensation.

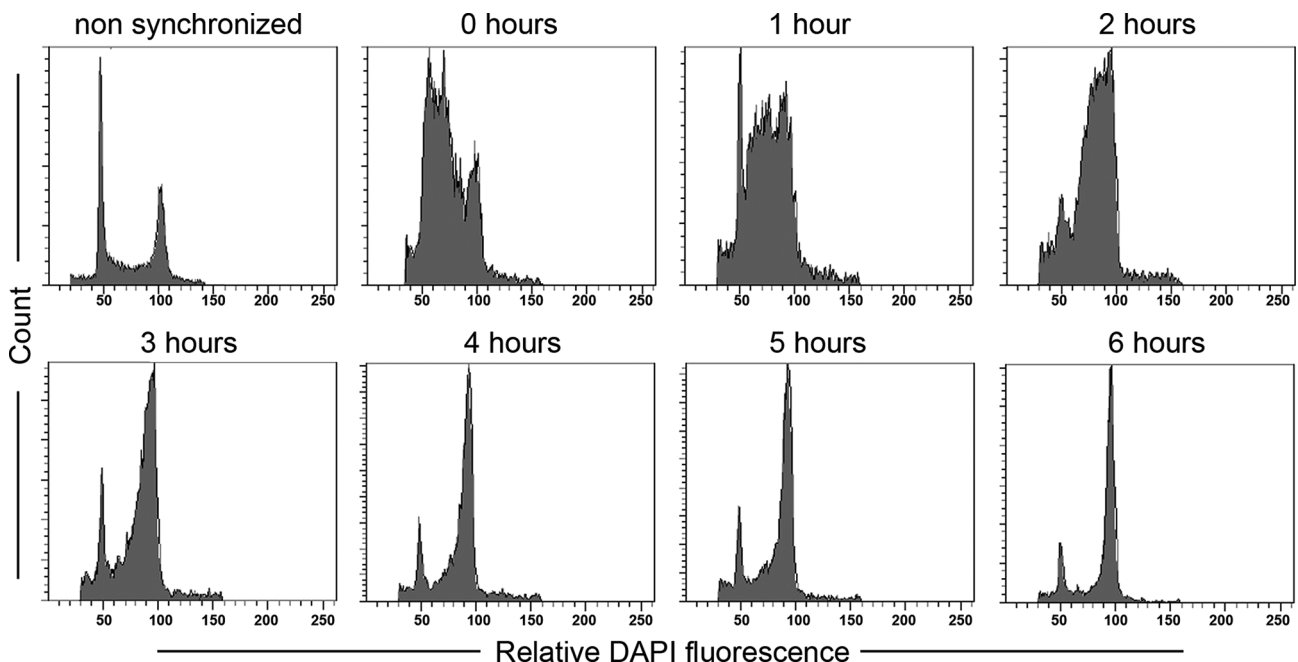


FIGURE 2 Cell cycle synchrony in root tip cells of barley (*Hordeum vulgare*) cv. Morex induced by a treatment with 2.0 mM hydroxyurea for 18 h. cell nuclei were isolated from root tips at different time points after a transfer to hydroxyurea-free nutrient medium and their DNA content was estimated by flow cytometry. The upper left histogram shows DNA content distribution in non-treated root tips. The sample taken at 0 h shows a large fraction of nuclei in S phase, indicating a precocious release from the hydroxyurea block. A majority of cells are at G2 phase at 6 h when they start entering mitosis

TABLE 1 Plant species in which chromosome suspensions used for flow cytometric analysis and sorting were prepared by mechanical homogenization of formaldehyde-fixed root tips

Species	Hydroxyurea		Recovery time	Metaphase accumulation		References (only the first reports are cited)
	Concentration (mM)	Duration (h)	Duration (h)	Chemical agent and concentration	Treatment duration (h)	
<i>Aegilops</i> spp. (<i>biuncialis</i> , <i>comosa</i> , <i>geniculata</i> , <i>umbellulata</i>)	1.25	18	5	APM 2.5 μ M	2 + ice overnight	[39]
<i>Aegilops</i> spp. (<i>cylindrica</i> , <i>markgrafii</i> , <i>triuncialis</i>)	1.25	18	5	APM 2.5 μ M	2 + ice overnight	[40]
<i>Aegilops</i> spp. (<i>speltooides</i> , <i>tauschii</i>)	1.25	18	5	APM 2.5 μ M	2 + ice overnight	[41]
<i>Agropyron cristatum</i>	1.25	18	4.5	APM 2.5 μ M	2 + ice overnight	[42]
<i>Arachis hypogea</i>	3.0	18	5	APM 5 μ M	2	[33]
<i>Asparagus officinalis</i>	3.0	18	6	APM 5 μ M	2	[43]
<i>Avena sativa</i>	2.0	18	4.5	Oryzalin 10 μ M	5	[33]
<i>Avena strigosa</i>	2.0	18	5	Oryzalin 5 μ M	2	Unpublished
<i>Cicer arietinum</i>	1.25	18	4	Oryzalin 5 μ M	2 + ice overnight	[44]
<i>Crocus sativus</i>	0.5	18	5	APM 5 μ M	3 + ice overnight	[33]
<i>Dasypyrum villosum</i> (syn. <i>Haynaldia villosa</i>)	1.25	18	5	APM 2.5 μ M	2 + ice overnight	[45]
<i>Elymus elongatum</i>	1.25	18	5	APM 2.5 μ M	2 + ice overnight	[33]
<i>Festuca pratensis</i>	1.0	18	4.5	N ₂ O (506.625 kPa)	2	[46]
<i>Gossypium hirsutum</i>	4.5	18	3.5	Oryzalin 10 μ M	2 + ice overnight	[33]
<i>Hordeum vulgare</i>	2.0	18	6.5	APM 2.5 μ M	2 + ice overnight	[47]
<i>Hordeum vulgare</i> cv. Barke	1.5	18	5.5	APM 2.5 μ M	2 + ice overnight	[33]
<i>Lens culinaris</i>	1.25	18	4.5	APM 10 μ M	2 + ice overnight	[33]
<i>Lolium perenne</i>	1.5	18	4.5	APM 5 μ M	2	[33]
<i>Lupinus angustifolius</i>	3.0	18	4	Oryzalin 2.5 μ M	2 + ice overnight	[33]
<i>Medicago sativa</i>	4.5	18	6	APM 2.5 μ M	3	[33]
<i>Pisum sativum</i>	1.25	18	4.5	APM 10 μ M	2 + ice overnight	[48]
<i>Rumex acetosa</i>	2.0	18	5	Oryzalin 10 μ M	2 + ice overnight	[33]
<i>Saccarum officinarum</i>	3.5	18	1.0	APM 2.5 μ M	3	[32]
<i>Secale cereale</i>	2.5	18	6.5	APM 10 μ M	2 + ice overnight	[49]
<i>Silene</i> spp. (<i>latifolia</i> , <i>dioica</i>)	2.0	18	5	oryzalin 2.5 μ M	2	[33]
<i>Triticum aestivum</i>	2.0	18	5.5	APM 2.5 μ M	2 + ice overnight	[33]
<i>Triticum monococcum</i>	1.25	18	5	APM 2.5 μ M	2 + ice overnight	[50]
<i>Triticum militinae</i>	2.0	18	5.5	APM 2.5 μ M	2 + ice overnight	[51]
<i>Triticum dicoccoides</i>	2.0	18	5.5	APM 2.5 μ M		[52]

(Continues)

TABLE 1 (Continued)

Species	Hydroxyurea		Recovery time	Metaphase accumulation		References (only the first reports are cited)
	Concentration (mM)	Duration (h)	Duration (h)	Chemical agent and concentration	Treatment duration (h)	
					2 + ice overnight	
<i>Triticum durum</i>	1.25	18	5	APM 2.5 μ M	2 + ice overnight	[53]
<i>Triticum timopheevii</i>	2.0	18	5.5	APM 2.5 μ M	2 + ice overnight	Unpublished
<i>Triticum urartu</i>	2.0	18	5.5	APM 2.5 μ M	2 + ice overnight	[41]
<i>Vicia faba</i>	1.25	18.5	4.5	APM 2.5 μ M	2	[29]
<i>Vicia sativa</i>	2.5	18.5	3.5	Oryzalin 5 μ M	2	[54]
<i>Vigna unguiculata</i>	1.25	18	4	Oryzalin 2.5 μ M	2 + ice overnight	[33]
<i>Zea mays</i>	3.0	18	3.5	N ₂ O 506.625 kPa	3	[33]

Abbreviation: APM, amiprofos-methyl.

2.3 | Release of intact mitotic chromosomes from cells

All protocols on the preparation of chromosome suspensions from synchronized root tip meristems published to date have been based on mechanical homogenization of root tips. Doležel et al. [29] showed that a mild fixation with formaldehyde prior to root tip chopping increases the yield of intact chromosomes. In the original protocol [29], root tips of *Vicia faba* were homogenized by chopping using a scalpel in a chromosome isolation buffer, and chromosome clumps were dispersed by syringing through a hypodermic needle. Although this procedure results in high quality chromosome suspensions, it is not practical for species with small roots and for experiments which require many samples. Thus, chromosome suspensions are almost universally prepared by homogenization of root tips using a mechanical homogenizer as described by Gualberti et al. [48]. Nevertheless, chopping can still be useful when handling delicate and/or rare samples.

In both versions of the protocol, formaldehyde fixation of root tips enhances the resistance of metaphase chromosomes to mechanical shearing during the isolation and subsequent flow cytometric analysis and sorting. To achieve the highest yield of intact chromosomes, the extent of the fixation, which is defined by formaldehyde concentration and length of the fixation, needs to be determined experimentally for each species by microscopic observation of crude chromosome suspensions and flow cytometric analysis of chromosome samples [29]. The observations of Lee et al. [56] indicate that it is possible to avoid the fixation step, however, at the expense of a much lower chromosome yield.

The chemical composition of chromosome isolation buffer is critical to ensure high chromosome yields and stabilize their morphology. The composition and pH must also be compatible with chromosome staining conditions, and depending on the use of sorted chromosomes

in downstream applications, the buffer must protect DNA and proteins from degradation. The polyamine-based LB01 buffer [58], proved to satisfy these requirements. However, it needs to be modified if high molecular weight (HMW) DNA is to be prepared from flow-sorted chromosomes [59], or if chromosomal proteins are isolated for proteomic analyses [60].

In general, isolated plant chromosomes are not stable over time, and therefore should be analyzed on the day of isolation. Stability can be species-specific: for example, our unpublished results indicate that chromosome suspensions prepared from formaldehyde-fixed roots of *Vicia faba* and *Pisum sativum* can be stored at 4°C for several months without detectable negative effects. On the other hand, cereal chromosomes deteriorate more rapidly and even 1 day-old chromosome samples display lower resolution of chromosome peaks or populations on flow karyotypes. It is worth mentioning that isolated wheat chromosomes denatured by sodium hydroxide, as done in the fluorescence in situ hybridization in suspension (FISHIS) protocol [61] are suitable for flow karyotyping for as long as several months. Moreover, the addition of hexylene glycol improved the long-term stability of *Vicia faba* chromosome suspensions and avoided chromosome clumping during storage [62].

3 | CHROMOSOME ANALYSIS (FLOW KARYOTYPING)

3.1 | Choice of instruments

Chromosome samples may be analyzed on any flow cytometer with optical setup suitable for the quantification of fluorochrome(s) used to label the isolated chromosomes. The ability to analyze light scatter is helpful, but the main threshold is settled on DNA fluorescence channel. The instrument must be stably aligned to achieve the highest

possible resolution during long runs. If the instrument has interchangeable flow chambers/nozzles, small orifices (typically 70 μm in diameter) are recommended as this results in higher resolution and chromosome discrimination. A simple sheath fluid, comprising 50 mM NaCl, is suitable for all chromosome analysis and sorting experiments (unpublished).

Prior to the analysis, chromosome samples are filtered through a nylon mesh (21–36 μm pore size) to eliminate larger particles. Flow cytometric chromosome analysis, or flow karyotyping, leads to chromosome classification according to their optical parameters. The most important has been fluorescence of dyes bound to DNA, and all fluorescence pulse parameters, including pulse height (FL-H), area (FL-A) and width (FL-W) are informative [63, 64]. In addition, light scattering properties are useful to gate out debris present in chromosome samples. Due to the poorer light collection properties of the first

generation jet-in-air flow cytometers and sorters, lasers with output power of several hundred milliwatt were needed to achieve the required sensitivity and resolution of fluorescence signals [62]. The newer generation of flow cytometers in which optical parameters are interrogated in an enclosed stream allows high resolution flow karyotyping with air-cooled compact solid state lasers output power of as low as 20 mW [64] (Figure 3).

3.2 | Univariate flow karyotyping

In single parameter, or univariate, flow karyotyping, chromosomes are classified according to fluorescence of a single fluorochrome bound to DNA, which is the most important signal in flow cytometric chromosome analysis. In addition, light scatter properties are analyzed and forward light

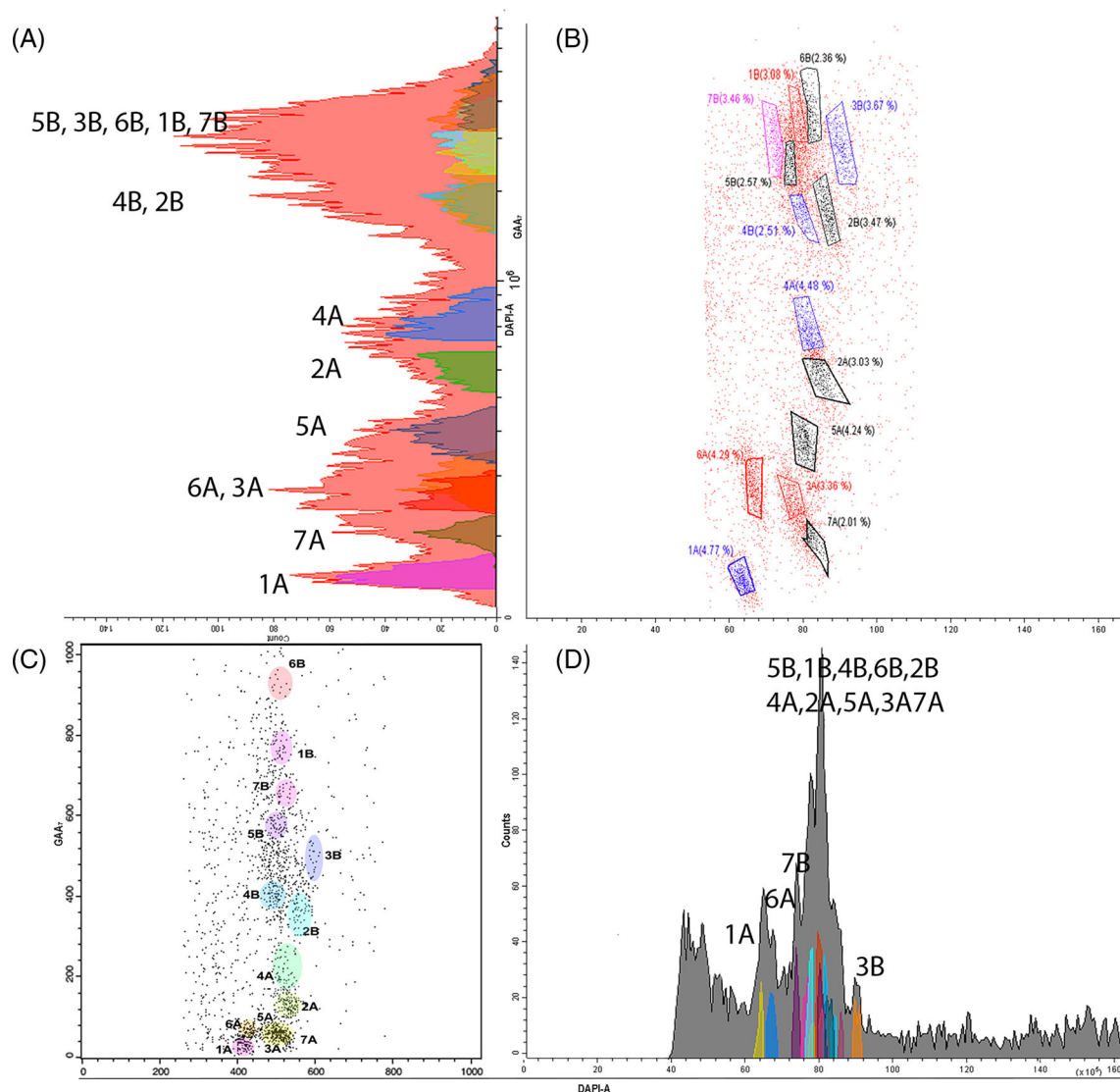


FIGURE 3 Comparison of bivariate flow karyotyping (GAA-FITC vs. DAPI) by two flow cytometers differing in optical design and laser output power. (A, B, C) BC Cytotflex S equipped with solid state low power lasers (375 nm at 20 mW and 561 nm at 30 mW). (D) BD FACS Vantage SE jet in air sorter equipped with two water-cooled argon lasers (357–361 nm at 200 mW and 488 nm at 400 mW). Chromosomes isolated from durum wheat (*Triticum durum*, $2n = 4x = 28$) cv. Cappelli were labeled by FISHIS using the same probe GAA₇ and counterstained by DAPI. The chromosome discriminative capability of the bivariate analysis is shown by single chromosome regions (in color)

scatter (FSC-A) is a recommended parameter to couple with DNA fluorescence pulses (FL-A) to discriminate small debris and chromosomes (Figure 4(A)). The analysis of chromosome fluorescence intensity (FL-A) results in histogram of relative fluorescence intensity, representing the univariate flow karyotype (Figure 4(B)). The ability to discriminate individual chromosomes within the flow karyotype depends on differences in chromosome DNA content, or DNA base content, among the chromosomes within karyotype and on the resolution of the analysis. The latter depends on: (i) the quality of the chromosome sample, which should contain the highest proportion of intact chromosomes and lowest of damaged chromosomes and chromosome clumps; (ii) the method used to stain chromosomal DNA; and (iii) the technical parameters of the instrument and quality of its alignment.

From the range of DNA binding fluorochromes that are available, only a few have been used for univariate flow karyotyping in plants. These included the DNA intercalators ethidium bromide [21] and propidium iodide [22], the fluorescent antibiotics mithramycin and chromomycin A3 [22], and DAPI, which binds preferentially to AT-rich regions of DNA [29]. The choice of a fluorochrome depends primarily on the available lasers and the optical setup of the instrument. Given the lack of comparative analyses, it seems that for native non-fixed chromosomes, any of the above-mentioned fluorochromes are useful. The situation is different for chromosomes isolated after formaldehyde fixation. Here, only DAPI is recommended as its binding is not affected by the fixation [62], most probably a consequence of the way DAPI binds to chromatin [65, 66].

3.3 | Bivariate flow karyotyping

In a majority of plant species analyzed so far, differences in relative DNA content, or DNA base content among the chromosomes, are too

small to permit discrimination of all chromosomes using a single DNA fluorochrome. Unfortunately, bivariate flow karyotyping after simultaneous chromosome staining with AT- and GC-binding fluorochromes, which is a standard in human flow karyotyping [67], does not lead to a significant improvement [68, 69] and has been rarely used. An alternative, and to date the only successful method for bivariate flow karyotyping in plants, has been to fluorescently label a repetitive DNA sequence which is abundant and non-homogenously distributed across the chromosomes of the karyotype.

First attempts to label particular DNA sequences of chromosomes in suspension by modified primed in situ DNA labeling (PRINS) [70] brought some promising results [71], but the method suffered from poor reproducibility and has not been used further. Afterwards, attempts to apply fluorescence in situ hybridization (FISH) to chromosomes in suspension were made and although Ma et al. [72] achieved chromosome labelling in suspension, the suitability of the samples for flow cytometry was not confirmed. The difficulties were finally overcome by Giorgi et al. [61], who developed a protocol for FISHIS, which is fully reproducible (Figure 4(C)). Their method differs from standard FISH protocols in that chromosomal DNA is denatured by alkali and no washing steps are performed, avoiding chromosome clumping. To date, the method has been used to label different microsatellite repeats on chromosomes in suspension from several plant species (Table 2). The probes are mainly fluorescent oligonucleotides, which do not require DNA denaturation prior to use. In some species, chromosome resolution can be further improved by using oligonucleotide probes for two different microsatellites [50, 73, 74]. Lucretti et al. [74] demonstrated that other repetitive sequences, such as the 18S-5.8S-26S ribosomal DNA [77] labeled by a fluorochrome by nick-translation, can also be used as probes for FISHIS. It is important that FISHIS can be easily integrated into chromosome sample preparation pipeline as it can be performed at room temperature in less than 2 h.

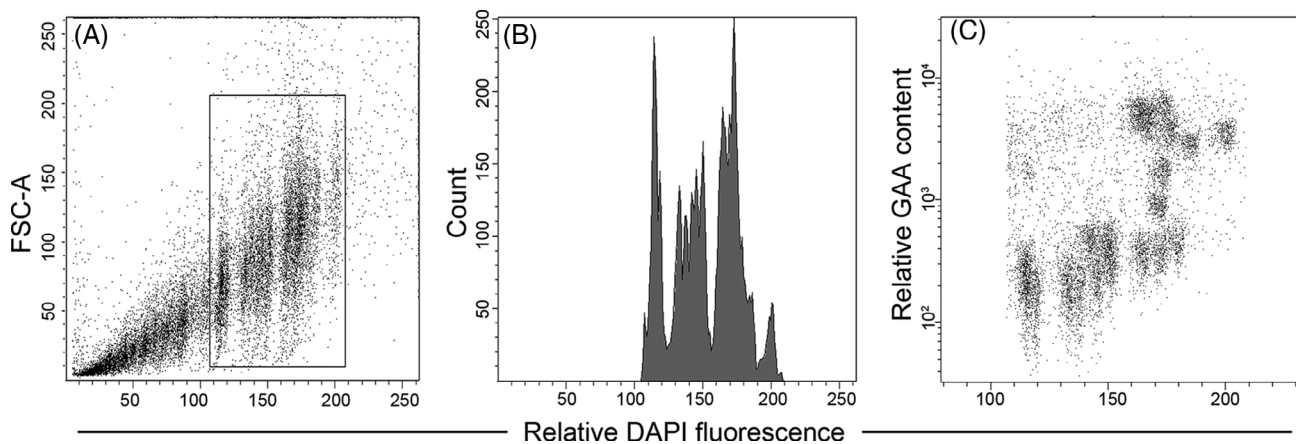


FIGURE 4 Flow cytometric chromosome analysis (flow karyotyping) in bread wheat (*Triticum aestivum*, $2n = 6x = 42$) cv. Chinese spring. (A) Dot-plot DAPI-A versus FSC-A is used to select the population of intact chromosomes and exclude small chromosome fragments and cell debris (gate window is shown as black rectangle). (B) Monovariate flow karyotype (DAPI-A) shows three composite peaks representing groups of chromosomes; only chromosome 3B is represented by a well discriminated peak visible on the right side of the distribution. (C) Bivariate flow karyotype GAA-FITC versus DAPI-A permits discrimination of almost all 21 chromosomes of bread wheat. Note that in panel (C) the Y axis is log scale to accommodate the large range of FITC fluorescence intensities after labelling GAA microsatellites by FISHIS

TABLE 2 Plant species for which chromosome labeling by FISHIS has been used for flow cytometric chromosome sorting

Species	Probe	Reference
<i>Aegilops biuncialis</i>	(GAA) ₇	Unpublished
<i>Aegilops comosa</i>	(GAA) ₇ + (ACG) ₇	[73]
<i>Aegilops geniculata</i>	(GAA) ₇	Unpublished
<i>Aegilops markgrafii</i>	(GAA) ₇ + (ACG) ₇	[73]
<i>Aegilops sharonensis</i>	(GAA) ₇	Unpublished
<i>Aegilops speltoides</i>	(GAA) ₇ + (ACG) ₇	[73]
<i>Aegilops triuncialis</i>	(GAA) ₇	Unpublished
<i>Aegilops umbellulata</i>	(GAA) ₇	[73]
<i>Aegilops uniaristata</i>	(GAA) ₇	Unpublished
<i>Agropyron cristatum</i>	(GAA) ₇	[42]
<i>Dasyphyrum villosum</i> (<i>H. villosa</i>)	(GAA) ₇	[61]
<i>Dasyphyrum villosum</i> (<i>H. villosa</i>)	pTa71 (rDNA)	[74]
<i>Triticum aestivum</i>	(GAA) ₇ (AG) ₁₂	[61]
<i>Triticum aestivum</i>	(GAA) ₇	[75]
<i>Triticum aestivum</i> (transgenic lines)	(GAA) ₇	[76]
<i>Triticum dicoccoides</i>	(GAA) ₇	[52]
<i>Triticum durum</i>	(AG) ₁₂ (AAT) ₇ (AAC) ₅	[61]
<i>Triticum durum</i>	(AG) ₁₂	[74]
<i>Triticum militinae</i>	(GAA) ₇	[51]
<i>Triticum monococcum</i>	(ACC) ₅ + (GAA) ₇	[50]
<i>Triticum timopheevii</i>	(GAA) ₇	Unpublished

3.4 | Flow karyotype description

Interpretation of flow karyotypes requires the assignment of chromosomes to individual peaks on histograms of fluorescence intensity, or in populations of bivariate flow karyotypes. This is a difficult task if the analysis is done using an instrument without a sorting option. In principle, one may compare the experimental data with theoretical models calculated based on relative chromosome lengths [78, 79] or even estimating the total fluorescence labeling intensities coupled with DNA content [80]. However, this is a rough approximation and differences in AT/GC content, and heterogeneity in chromatin condensation among chromosomes in a karyotype may cause differences between theoretical and experimental flow karyotypes [81]. The task is somewhat easier if one or more chromosomes differ significantly in size, or AT/GC content from other chromosomes. Such situation may be observed in chromosome deletion, translocation, or alien addition lines [62]. However, the only truly reliable way to assign chromosomes to peaks or populations on a flow karyotype is to sort chromosomes and identify them after microscopic observation, or using PCR with specific primers, or by sequencing. Microscopic observation is the preferred method, since it allows a detailed characterization of the sorted fraction by determining the presence of individual chromosome types [82].

4 | CHROMOSOME SORTING

4.1 | Chromosome population gating and sort windows

There are two basic applications of chromosome sorting. The first is the purification of mitotic chromosomes in bulk to study their molecular organization [83, 84], prepare microscopic slides for mapping DNA sequences by FISH at high spatial resolution [85, 86], and localize chromosomal proteins by immunocytochemical methods [87, 88]. The second, and by far more frequent application, has been the purification of particular chromosomes to dissect nuclear genomes to chromosomes, or small groups of chromosomes, to aid in genome sequencing [16–18], targeted development of DNA markers [8–11], and gene cloning [14, 15, 89–92].

To discriminate a particular population of chromosomes from other particles in the sample, optical parameters which define the population of interest are selected. In monovariate flow karyotyping, it is advisable first to electronically filter out small debris by setting a gate on a dot-plot FSC-A versus DAPI FL-A, and then define the region of interest on a dot-plot DAPI FL-A versus DAPI FL-W (Figure 5(A)). Setting the sort windows may be easier on bivariate flow karyotypes, such as DAPI FL-A versus GAA-FITC FL-H, where the chromosome populations can be more cleanly separated (Figure 5(B)). The definition of sort windows is critical since these determine the chromosomes that are sorted and affect the degree of contamination of the sorted fractions by other chromosomes, chromosome clumps and fragments.

4.2 | Discrimination and sorting chromosomes of interest

The karyotypes of some plant species comprise two or more chromosomes that are similar in size and DNA repeat content, and currently available methods for fluorescent staining of chromosomal DNA or labeling particular DNA repeats may fail to discriminate between these chromosomes. The consequence is the appearance of composite peaks on monovariate flow karyotypes, or mixed populations on bivariate flow karyotypes. The extent of the problem varies between species, from the ability to discriminate all chromosomes using a single FISHIS probe, such as in *Haynaldia villosa* [61] and some *Aegilops* species [73] (see Table 2 for the list of probes used for FISHIS), to the ability to discriminate only a few chromosomes even after FISHIS labeling, such as in *Secale cereale*. There is a clear effect of polymorphisms in DNA repeat content, and whereas in some *Hordeum vulgare* cultivars only five of the seven chromosomes can be resolved, all seven chromosomes can be distinguished in other cultivars.

In polyploids, and in bread wheat in particular, one solution has been to use lines with chromosome deletions, such as the telocentric stocks [3]. In polyploids, it is also possible to develop alien chromosome addition lines (Figure 6). Thus, wheat-rye chromosome addition lines were used to purify rye chromosomes for shotgun sequencing and draft genome assembly [16], and wheat-barley telosome addition

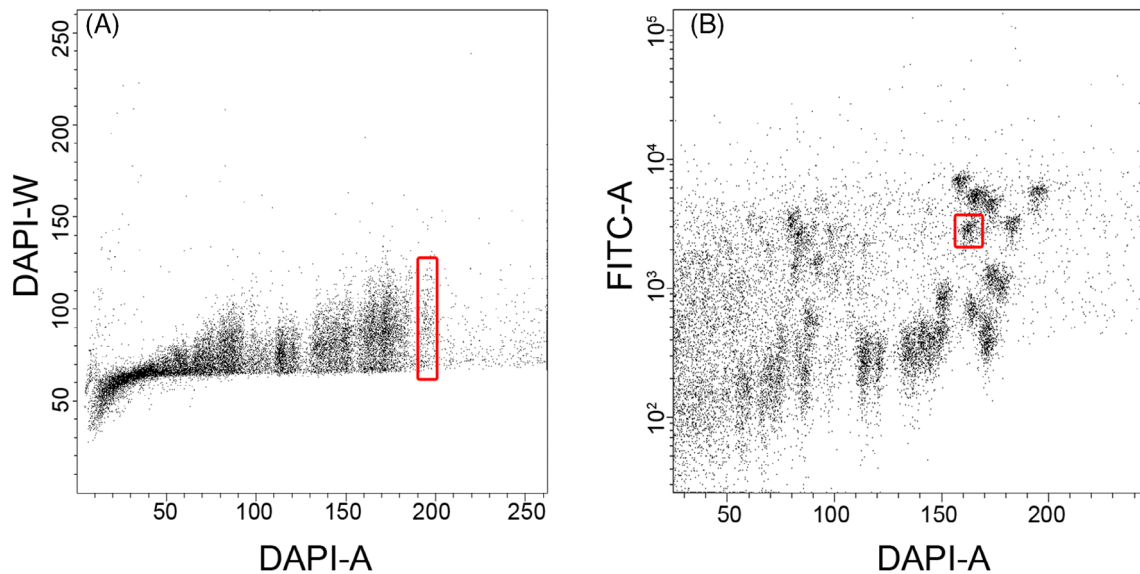


FIGURE 5 Setting sort windows (red rectangles) to isolate particular chromosomes from bread wheat (*Triticum aestivum*, $2n = 6x = 42$) cv. Chinese Spring. (A) In monovariate flow karyotyping, when chromosomes are stained just by DAPI, sort window is set on a dot-plot DAPI-A versus DAPI-W. The example is given for sorting the largest chromosome 3B. (B) Bivariate flow karyotyping DAPI-A versus GAA-FITC allows discrimination of chromosomes that cannot be discriminated using the monovariate analysis. The window is set to sort chromosome 5B, which is included in the large composite peak representing 10 different chromosomes on a monovariate flow karyotype [Color figure can be viewed at wileyonlinelibrary.com]

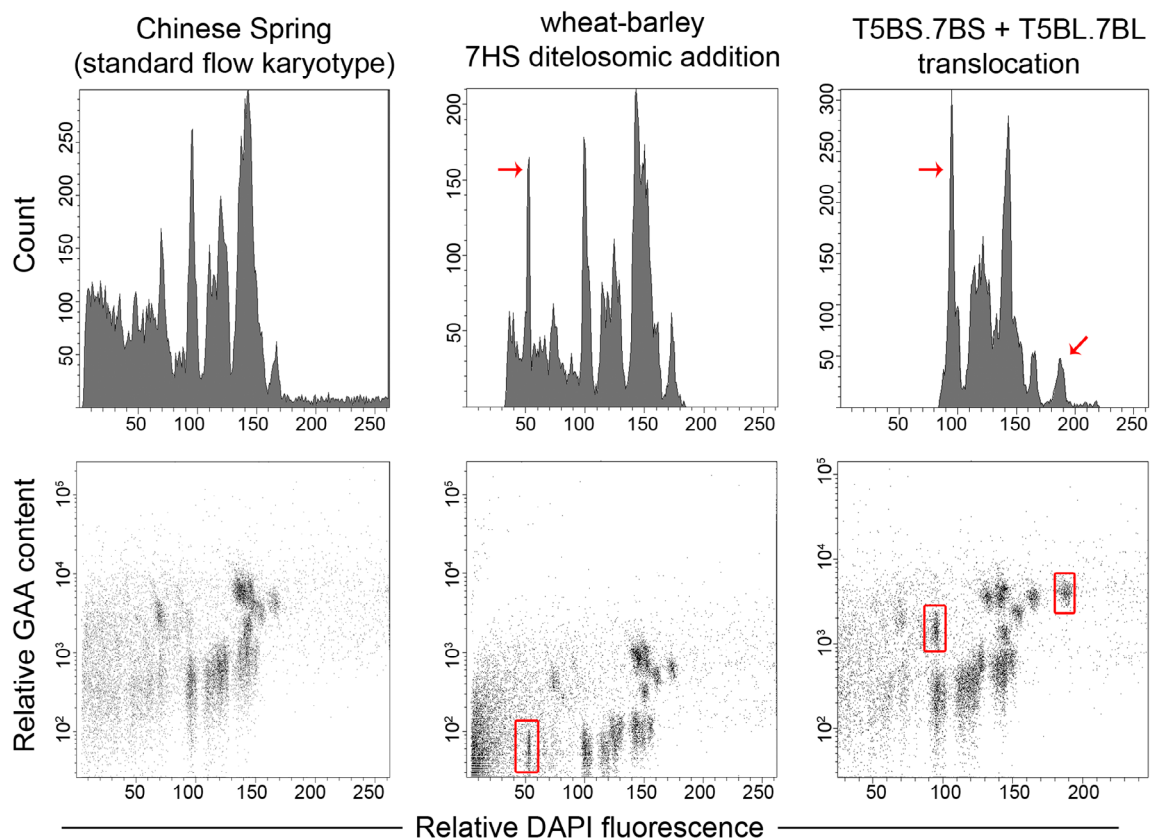


FIGURE 6 Chromosome discrimination in bread wheat (*Triticum aestivum*, $2n = 6x = 42$) cv. Chinese Spring with a wild-type karyotype (left column), wheat-barley 7HS ditelosomic addition line (middle column) and wheat chromosome translocation line T5BS.7BS + T5BL.7BL (right column). The upper row shows monovariate flow karyotypes obtained after the analysis of DAPI fluorescence pulse area (DAPI-A) and red arrows point to peaks representing chromosomes, which can be easily discriminated. The lower row shows bivariate flow karyotypes DAPI-A versus GAA-FITC and red rectangles define the populations of chromosomes represented by peaks in monovariate flow karyotypes (upper row). Note that the small translocation chromosome T5BS.7BS is included in the composite peak of wild-type chromosomes 1D, 4D, and 6D on a monovariate flow karyotype, while it is clearly discriminated in the bivariate flow karyotype [Color figure can be viewed at wileyonlinelibrary.com]

lines facilitated isolation of barley chromosome arms for SNP mapping using DNA arrays [93] and sequencing to obtain a high resolution sequence-based gene map of the crop [17]. Certain translocations may alter chromosome size, and, as they may be induced also in diploids, provide an attractive way to discriminate particular chromosomes [47, 62, 63, 94]. However, the development of such lines is time-consuming, and they cannot be easily obtained. Although the sorted translocation chromosomes facilitate gene mapping [47, 94, 95], their DNA may not be directly suitable for other purposes, such as the genome sequence assembly.

If the chromosome of interest cannot be distinguished by any of the above-mentioned strategies, the option is to sort single copies of chromosomes from a composite peak/population into a set of PCR tubes, or wells in a microtitre plate, and amplify DNA of single chromosomes to produce microgram amounts of DNA [96]. As anonymous chromosomes are sorted, their identity is revealed only after amplification using specific primers or shotgun sequencing. Although the spectrum of applications may be limited due to variable DNA fragment lengths, ranging from 300 bp to 20 kb, and by amplification that is not fully representative [96], the approach has been used recently to validate reference genome assembly of garden pea and reveal chromosome translocations in its wild relatives [19].

4.3 | The identity of sorted chromosomes and the purity in sorted fractions

The observation of sorted fractions by fluorescence microscopy is strongly recommended to assign chromosomes to populations within a flow karyotype, confirming that a chromosome of interest is sorted, and to identify other chromosomes or fragments that contaminate the sorted fraction. To do this, about 1000 chromosomes are sorted onto

a microscopic slide into a small drop of chromosome isolation buffer LBO1 supplemented with 5% (w/v) sucrose to preserve chromosome morphology during air-drying [81]. The slides prepared this way are used for FISH with probes that enable identification of individual chromosomes within a karyotype (Figure 7). The added bonus of labelling chromosomes by FISH prior to sorting is that the chromosomes sorted onto a microscope slide do not have to be labeled by FISH and are directly suitable for fluorescence microscopy as the fluorescent labels are not removed during the sorting. Evaluation of a few hundred chromosomes is sufficient to characterize the population of sorted chromosomes.

This approach is feasible only with droplet sorters as the volume droplets in which the particles are sorted is small (e.g., 1 nl) and chromosomes are spread across a small area on the slide making microscopic observation easier. In principle, fluidic switch sorters could also be used. However, as the volume of the sorted fraction is much larger, it is necessary to pellet the chromosomes before transferring them onto a slide, and the centrifugation can result in chromosome loss and clumping.

4.4 | The yield of sorted chromosomes

As in other applications of flow cytometric sorting, chromosome sort purity and yield are inversely proportional, and setting up a very tight sort window may lead to higher purity, but also low sort rates. It is thus critical to run chromosome samples at rates securing high resolution of DNA peaks/chromosome populations. When analyzing chromosome samples prepared from bread wheat according to Vrána et al. [81], the recommend sample rate is about 2000 particles/s. The exact size, shape, and position of the sort window needs to be determined experimentally, relying on the results of microscopic observation of

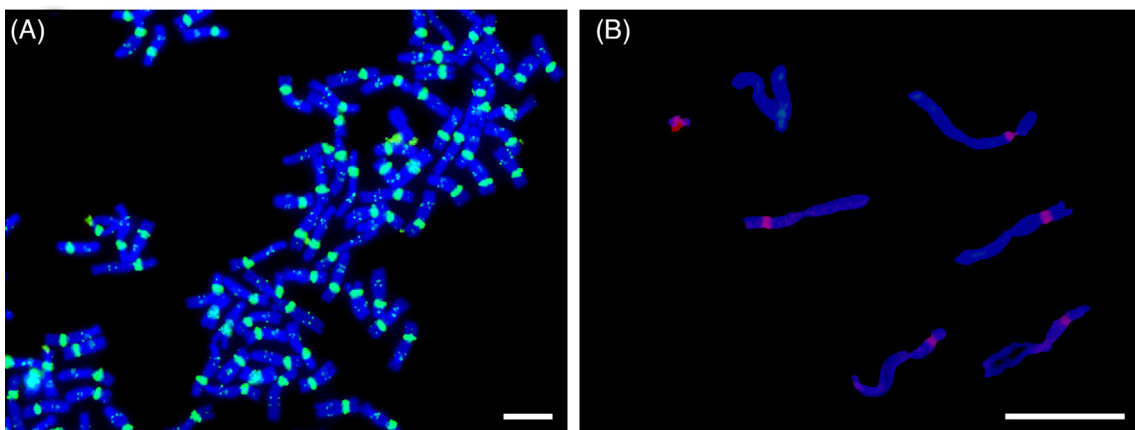


FIGURE 7 Images of chromosomes flow-sorted onto microscope slides. (A) Chromosomes 5B sorted from bread wheat (*Triticum aestivum*) cv. Chinese spring. (B) Chromosomes 1B and 6B bearing NOR loci sorted from durum wheat (*Triticum durum*) cv. Creso. Prior to analysis and sorting, chromosomes in suspension were labeled by FISHIS: (A) using a probe (GAA)₇-Alexa488 (yellow-green), and (B) using a probe for pTa71-Cy3 (red). Note that during landing on a slide, a satellite has been lost from the chromosome on the top left side. In both cases, chromosomes were stained by DAPI (blue). The specific fluorescent labeling patterns obtained after FISHIS remain stable during the sorting and facilitate identification of the sorted chromosomes. Bars = 10 μ m

flow-sorted particles. In a typical experiment, a chromosome of bread wheat can be sorted from a sample prepared according to Vrána et al. [81] at rates of 5–10 particles/s. In order to keep the final volume of the sorted fraction low, the sorting is done using the single-droplet mode.

5 | CHROMOSOME SORTING FOR VARIOUS APPLICATIONS

Plant chromosomes sorted by flow cytometry have been used in a variety of applications, including molecular cytogenetics, molecular biology, genome sequencing, and gene cloning, and proteomics [36]. The way chromosomes are sorted for each application may differ as outlined below.

5.1 | Preparation of high molecular weight DNA for BAC library construction, optical mapping and long-read DNA sequencing

In order to maintain chromosomal DNA integrity, chromosomes should be isolated into a buffer optimized for this purpose [59]. As these applications require hundreds of nanograms to several micrograms of DNA, a large number of chromosomes (typically 10^5 – 10^6) must be sorted. When determining the number of chromosomes to be sorted, one needs to consider the molecular size of the sorted chromosome, the fact the mitotic chromosomes have two chromatids, and also the losses during the preparation of HMW DNA from sorted chromosomes, which may reach 75%–80%. In bread wheat, a typical sort yield is 400,000–900,000 chromosomes per working day, hence the preparation of HMW DNA takes days or even weeks, and many chromosome samples will need to be prepared. The chromosomes are sorted into 1.5-ml DNA low-binding polystyrene tubes in batches, ranging from 200,000 to 700,000 for bacterial artificial chromosome (BAC) library construction and optical mapping, respectively. It is recommended to sort into 1.5× chromosome isolation buffer (220 μ l for BAC library construction and 770 μ l for optical mapping) to counterbalance dilution by sheath fluid.

5.2 | Sequencing amplified chromosomal DNA

Many applications in molecular biology and genomics do not require high molecular weight DNA, and DNA samples with fragments sizes up to 20 kb are sufficient. This offers the possibility to employ whole genome amplification of DNA to produce microgram amounts of DNA from a smaller number of chromosomes [97]. The number of chromosomes that need to be sorted depends on chromosome size, being determined such that the equivalent of 40 ng sorted DNA is obtained. In case of bread wheat, this corresponds to about 20–30 thousand chromosomes, depending on chromosome size. The chromosomes are sorted into 0.5-ml PCR tubes containing 40 μ l sterile deionized water.

The tubes with chromosomes are spun down using a microcentrifuge immediately after sorting and kept at -20°C until used for DNA amplification.

5.3 | Sequencing non-amplified chromosomal DNA

The introduction of protocols for Illumina sequencing libraries requiring nanogram amounts of DNA or less, made it possible to skip the chromosome amplification step. In fact, sequencing non-amplified chromosomal DNA results in better assemblies compared to those from amplified DNA [90]. The number of sorted chromosomes and the way they are collected is the same as in the protocol for sequencing amplified DNA. The tubes with chromosomes are spun down using a microcentrifuge immediately after sorting and kept at -20°C until used for preparation of DNA sequencing libraries.

5.4 | Single copy chromosome DNA sequencing

As minute amounts of DNA are amplified in this case, special precautions need to be taken to avoid sample contamination by DNA of other organisms and the samples should be processed under sterile conditions in a laminar flow hood. Single chromosomes are sorted into 3 μ l whole genome amplification mix in 0.2 ml PCR tubes, which are sterilized by autoclaving [96]. To prepare a positive control, 1000 chromosomes are sorted into another tube. A negative control is the 0.2 ml PCR tube with the amplification mix and without chromosomes. All tubes with chromosomes should be spun down using a microcentrifuge immediately after sorting.

5.5 | Chromosome conformation capture (Hi-C)

Chromosome flow-sorting can be used to study chromatin conformation by inferring its spatial contacts in all-versus-all manner using a chromosome conformation capture method termed Hi-C [98]. As the formaldehyde fixation is a crucial step in this analysis and directly impacts sequencing results, it needs to be optimized and usually slightly differs from the standard protocols used to prepare suspensions of intact chromosomes. In order to obtain sufficient number of contacts between DNA loci, 3–5 million chromosomes per replicate are sorted into 15 ml protein low-binding tubes with 2 ml LB01 buffer, which are kept on ice during the entire sorting procedure.

5.6 | Proteomic analysis

Proteomic analysis requires that proteins of flow-sorted chromosomes are protected from degradation. This is achieved by using a modified chromosome isolation buffer and a modified buffer into which the chromosomes are sorted. In order to improve the accessibility of chromosomal proteins, it is also recommended to reduce the extent of

formaldehyde fixation of synchronized root tips. This application has been developed to study protein composition of mitotic metaphase chromosomes [60] and all chromosomes of a plant are sorted, avoiding the need to discriminate particular chromosome type. The sample rate may thus be higher, reaching 6000 chromosomes/s, and sort yields range between 3.5 and 6.6 million chromosomes/working day. In order to isolate enough proteins for mass spectrometry, 5–10 million sorted chromosomes are required. To accommodate the high number of droplets with sorted chromosomes, sorting is done into 5 ml polystyrene tubes, containing 1 ml of modified chromosome isolation buffer.

6 | BEST PRACTICES

- The most suitable material for preparing liquid chromosome suspensions are meristem tips of actively growing roots of young seedlings, bulbs, or stem sections.
- When setting-up a methodology for a new species, sufficient amount of plant material (seeds, bulbs, or stem sections) should be available to allow for a thorough optimization of individual steps of the protocol.
- To prepare liquid chromosome suspensions suitable for flow cytometry, synchronized root tips should contain at least 40% of cells accumulated at mitotic metaphase.
- The use of DNA low-binding tubes is recommended for sample preparation and storage.
- Mild fixation of plant material by formaldehyde prior to mechanical homogenization increases chromosome yields, preserves chromosome morphology and improves their mechanical stability.
- To stain DNA of chromosomes isolated after formaldehyde fixation, only DAPI is recommended and the instrument should be equipped with UV, near-UV, or violet laser for optimal DAPI excitation.
- The flow sorter must be precisely aligned to achieve high resolution in DNA fluorochrome channel(s) with the coefficient of variation of chromosome peaks as low as possible (ideally less than 2%). The instrument alignment must be stable during sort runs.
- The chromosome content of populations identified by flow karyotyping and the purity at which they can be flow-sorted should be checked by microscopic observation of the populations sorted onto a microscopic slide. The check should be repeated regularly during long sort runs.
- Bivariate flow karyotyping DAPI fluorescence versus fluorescence of FISHIS-labeled DNA repeat(s) should always be tested to verify if it helps to improve the discrimination of chromosome populations.
- DNA probes suitable for FISHIS need to be selected empirically for each new species. In order to achieve a specific and intensive FISHIS labelling, pH and time of the denaturation step as well as final pH of the liquid chromosome suspension after stopping the denaturation must be carefully controlled.

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CONFLICT OF INTEREST

The authors declared no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Jaroslav Doležel: Conceptualization; funding acquisition; supervision; writing-original draft; writing-review & editing. **Sergio Lucretti:** Conceptualization; funding acquisition; project administration; writing-original draft; writing-review & editing. **István Molnár:** Visualization; writing-review & editing. **Petr Cápál:** Visualization; writing-review & editing. **Debora Giorgi:** Visualization; writing-original draft; writing-review & editing.

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