Theor Appl Genet (1991) 82:101–111

THEORETICAL AND APPLIED GENETICS

Preparation and flow cytometric analysis of metaphase chromosomes of tomato

K. Arumuganathan [1], J. P. Slattery [2], S. D. Tanksley [1] and E. D. Earle [1,*]

[1] Department of Plant Breeding and Biometry, Cornell University, Ithaca, NY 14853-1902, USA
[2] Flow Cytometry and Video Microscopy facility, Cornell University, Ithaca, NY 14853, USA

Received August 27, 1990; Accepted November 8, 1990
Communicated by P. Maliga

Summary. A procedure for the preparation of tomato chromosome suspensions suitable for flow cytometric analysis is described. Rapidly growing cell suspension cultures of *Lycopersicon esculentum* cv VFNT cherry and *L. pennellii* LA716 were treated with colchicine to enrich for metaphase chromosomes. Metaphase indices between 20 and 35% were routinely obtained when cultures were exposed to 0.1% colchicine for 15–18 h after 2 days of subculture. Mitotic cells were isolated by brief treatment with cell wall digesting enzymes in a medium with low osmolarity (~325 mOsm/kg of H_2O). The low osmolarity medium was needed to avoid the chromosome clumping and decondensation seen in standard media. Suspensions of intact chromosomes were prepared by lysing swollen protoplasts in various buffers ($MgSO_4$, polyamines, hexylene glycol, or KCl-propidium iodide) similar in contents to the buffers used to isolate mammalian chromosomes. For univariate flow cytometric analysis, chromosome suspensions were stained with a fluorescent DNA-binding stain (propidium iodide, Hoechst 33258, mithramycin, or chromomycin A3) and analyzed using an EPICS flow cytometer (Profile Analyzer or 753). Peaks for the chromosomes, chromatids, clumps of chromosomes, nuclei, and fluorescent debris were seen on a histogram of log of fluorescence intensity, and were confirmed by microscopic examination of the objects collected by flow-sorting. Chromosome suspensions prepared in $MgSO_4$ buffer have the highest frequency of intact chromosomes and the least fluorescent cellular debris. Peaks similar to theoretical univariate flow karyotypes of tomato chromosomes were seen on the observed univariate flow karyotypes, but were not as well resolved. Bivariate flow analysis of tomato chromosome suspension using double-stain combination, Hoechst 33258 and chromomycin A3, and two laser beams showed better resolution of some chromosomes.

Key words: Tomato chromosome suspension – Flow cytometry – Univariate analysis – Bivariate analysis – Chromosome sorting

Introduction

Preparation of suspensions of intact metaphase chromosomes followed by collection of single chromosome types by fluorescence-activated flow-sorting has been useful for construction of chromosome-specific libraries and gene mapping of the human genome (Gray and Langlois 1986; Van Dilla and Deaven 1990). We are interested in developing analogous procedures for tomato chromosomes to aid in mapping, isolation, and transfer of important plant genes.

Chromosome-specific or chromosome-enriched libraries would facilitate isolation of agriculturally important plant genes for which no protein product is known. These include genes for disease, insect, and stress resistance, as well as genes underlying quantitatively inherited characters (QTLs). If each of the tomato chromosomes could be purified by flow cytometry, it would be possible to construct chromosome-specific libraries of clones useful in gene isolation by chromosome walking. Selecting clones from a chromosome-specific library (corresponding to the chromosome on which the target gene is located) should be approximately 12-fold more productive than screening random libraries.

In contrast to the substantial body of work on mammalian chromosomes, experimental studies with isolated plant chromosomes are limited. Several authors have de-

* To whom correspondence should be addressed

scribed methods for isolating chromosomes from cell suspensions, root tips, or microsporocytes of various plant species (Malmberg and Griesbach 1980; Griesbach et al. 1982; Hadlaczky et al. 1983; Matthews 1983; de Laat and Blaas 1984; Conia et al. 1987). Published work on flow cytometric characterization and sorting of plant chromosomes has focused on three species, *Haplopappus gracilis* (de Laat and Blaas 1984), *Petunia hybrida* (Conia et al. 1987, 1988), and *Nicotiana plumbaginifolia* (Conia et al. 1989), but not on any major crop plant. Obtaining high quality chromosome samples suitable for flow cytometry has been very difficult. This requires not only that a sufficient number of cells be arrested in metaphase, but also that preparations be free of contaminating debris that confounds the analysis. The presence of cell walls on plant cells, as well as plastids, starch grains, vacuoles, etc. within them, makes the sample preparation more difficult than for animal cells. Suspensions of chromosomes that appear to have normal morphology by microscopic examination may still not be suitable for flow cytometry (Conia et al. 1987). This makes optimization of chromosome isolation procedures more laborious, since effects of alterations in isolation media must be tested by actual flow analysis.

This report describes a reliable procedure for obtaining large numbers of metaphase chromosomes in suspension, suitable for flow cytometry, from various tomato cell culture lines. Stains that show little nucleotide preference (e.g., propidium iodide), as well as stains that preferentially bind to AT-rich regions (e.g., Hoechst 33258) or to GC-rich regions (e.g., mithramycin, chromomycin A3) (Gray and Langlois 1986), were used for univariate flow cytometric analysis. To our knowledge, this is the first report of the univariate flow analysis of chromosomes of a crop plant. We also present a first report of bivariate flow analysis of plant chromosomes using a double-stain combination, Hoechst 33258 and chromomycin A3, and two laser beams. In the bivariate karyotype, tomato chromosome types are resolved based on the differences in base composition and DNA content. The tomato flow karyotypes will be useful for sorting chromosomes to construct chromosome-specific or chromosome-enriched DNA libraries.

Materials and methods

Cell lines and culture conditions

Tomato chromosome suspensions were prepared from cell suspension cultures of (1) *Lycopersicon esculentum* Mill. cv VFNT cherry (both a cell line obtained from Dr. J. Steffens, Cornell University, and freshly established cell lines), and (2) *Lycopersicon pennellii* Corr. P.I. 246502 (LA716), a green-fruited wild relative of cultivated tomato collected in Atico, Peru, and obtained from Tomato Genetics Cooperative (University of California, Davis). *Lycopersicon* species have a haploid chromosome number of 12; however, cells in culture became polyploid within about 3 months and had 48–96 chromosomes per cell.

Cell suspension cultures were established from rapidly growing, soft white calli initiated from root-hypocotyl explants of seedlings. The seeds were surface sterilized in 10% commercial bleach and washed three times in sterile distilled water. The seeds were germinated either on hormone-free MS medium (Murashige and Skoog 1962) solidified with 0.22% Gelrite or directly on callus-inducing medium. The medium for callus initiation and culture and cell suspension cultures consisted of macronutrients, micronutrients, and myo-inositol according to Murashige and Skoog (1962), vitamins according to Nitsch (1969), and 3% sucrose, pH 5.7 (DuPont et al. 1985). The medium was supplemented with growth regulators of either 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) and 1 mg/l 2iP (N^6[2-Isopentenyl]adenine) or 0.5 mg/l 2,4-D, 0.3 mg/l kinetin, and 5 mg/l indole-3-acetic acid (Malmberg and Griesbach 1980).

Suspension cultures were grown in 250-ml Erlenmeyer flasks on a rotary shaker (100 rpm) in 50 ml culture medium at 25 °C in the dark. They were subcultured every 7 days, when they were in early stationary phase of growth by diluting, (1:4) with fresh medium. Growth rate of the cell cultures was determined according to the method of DuPont et al. (1985), by calculating the ratio of settled cell volume to total volume (SCM) at 24 h intervals, using either side-arm flasks or graduated centrifuge tubes. Cells were allowed to settle for 15 min to measure the settled cell volume. In some experiments, in order to maintain uniform cell density, suspension cultures were subcultured by diluting 5 ml of settled cells into 50 ml total medium (SCM = 0.1).

Procedure for collecting large number of cells at metaphase

Two days after subculture, cells were exposed to colchicine (Sigma Co., St. Louis/MO) to accumulate a large number of cells at metaphase. Colchicine, freshly dissolved in culture medium (10 mg/ml), was added to the cell suspensions to a final concentration of 1 mg/ml (0.1%). Cells were incubated in the dark at 25 °C for 15–18 h.

Metaphase index (number of cells at metaphase × 100/total number of cells) was determined after staining the cells in a modified carbol fuchsin solution (Kao 1982). One drop of cell suspension was mixed with one drop of modified carbol fuchsin on a slide, and was warmed by passing it over an alcohol lamp several times. Stained cells were then flattened by pressing the slide (covered with a coverslip) between several layers of paper towelling. Tomato cells could be flattened easily without treating with wall-degrading enzymes, since they have thin cell walls and occur in small clumps in suspension culture. At least 250 cells were observed under the microscope to estimate the metaphase index.

Protoplasts were isolated from the cell suspension cultures with high metaphase index, in culture medium supplemented with 60 m*M* KCl (to adjust the osmolarity to about 325 mOsm/kg H_2O), 2% cellulysin (Calbiochem, La Jolla/CA), 1% macerase (Calbiochem, La Jolla/CA), and 0.1% pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Tokyo). In some experiments, different levels of KCl were added to the culture medium to find a suitable osmolarity for protoplast isolation (Table 1). A standard protoplast isolation medium (Tan et al. 1987), which contained 0.5 *M* mannitol as an osmoticum, was initially used for protoplast isolation but was later found to be unsuitable, since its high osmolarity (> 700 mOsm/kg H_2O) causes clumping of metaphase chromosomes.

Aliquots of 3 ml of cell suspension were mixed with 3 ml of culture medium containing 4% cellulysin, 2% macerase, 0.2% pectolyase, and 120 m*M* KCl and incubated for 1 h at

30 °C on a shaker at 50 rpm. The digestion mixture was then diluted with 6 ml of washing buffer (150 m*M* KCl, 5 m*M* hepes, pH 8.0, ~290 mOsm/kg of H_2O) and centrifuged at 500 rpm (~40 *g*) for 2 min. The pellet was resuspended in 6 ml of washing buffer and passed through 80-µm nylon mesh. The filtrate was collected directly in a 15-ml disposable centrifuge tube and diluted with 6 ml of washing buffer. The protoplasts were pelleted by centrifuging at 500 rpm for 2 min and kept on ice.

Chromosome isolation procedure

Protoplasts were resuspended in 12 ml of ice-old hypotonic buffer and immediately centrifuged at 500 rpm for 2 min. The hypotonic buffer was usually the $MgSO_4$ buffer (10 m*M* $MgSO_4$, 50 m*M* KCl, 5 m*M* hepes, 3 m*M* dithiothreitol, pH 8.0) (van den Engh et al. 1984), but a solution of 75 m*M* KCl was used when other chromosome isolation buffers [polyamines (Sillar and Young 1981; de Laat and Blaas 1984), hexylene glycol (Wray and Stubblefield 1970; Hadlaczky et al. 1983), KCl-propidium iodide (Buys et al. 1982)] or tomato cell culture medium without growth regulators were investigated for the preparation of chromosome suspensions. One milliliter of the ice-cold $MgSO_4$ buffer (or other chromosome isolation buffer) was added to the protoplast pellet, and the protoplasts were allowed to swell for 10 min at room temperature. Triton X-100 was then added to a final concentration of 0.25% [25 µl 10% (w/v) Triton X-100 per ml]. After 10 min incubation with Triton X-100 at room temperature, the protoplasts were mechanically ruptured by drawing the suspension into a sterile Pasteur pipette and extruding it forcefully from the pipette, with the stream directed along the side of the tube. This process was repeated five times. The resulting chromosome suspension was filtered through 33-µm nylon mesh or one layer of miracloth, to remove large particles which might clog the nozzle of the flow cytometer. The preparation was kept on ice and stained with DNA-specific dye(s). Flow cytometry was performed on the same day of preparation.

Univariate flow cytometric chromosome analysis

For univariate flow karyotype analysis, chromosomes were stained with a single dye at least 30 min before analysis. Propidium iodide (PI), mithramycin (MITH), chromomycin A3 (CA3), and Hoechst 33258 (HO) were used at concentrations of 25 µg/ml, 12.5 µg/ml, and 1.25 µg/ml, respectively. Stock solutions of PI (5.0 mg/ml) and HO (0.5 mg/ml) were made in sterile distilled water, and the stock solutions of MITH or CA3 (0.5 mg/ml were prepared freshly in $MgSO_4$ buffer.

Flow cytometric analyses of chromosome preparations stained with PI were performed with an EPICS Profile Flow Cytometer and/or an EPICS 753 Flow Cytometer (Coulter Electronics, Hialeah/FL). Analysis of MITH- and HO-stained samples was done with the EPICS 753 Flow Cytometer. PI-stained samples were excited with an Argon ion laser, all lines at 15 mW (Profile, Omnichrome 150) or at 488 nm, 500 mW (EPICS 753, Coherent Innova 90-5), and the red fluorescence of the PI-DNA complex was collected through a 457–502 nm laser blocking filter and a 590 or 610 nm long pass absorbance filter. Samples stained with MITH or CA3 were excited at 457 nm (100 mW), and the yellow fluorescence of MITH-DNA or CA3-DNA complex collected through a 457-nm laser blocking filter and a 550 nm long pass dichroic filter. HO-stained samples were excited in the UV region (351 and 364 nm, 100 mW), and the blue fluorescence of HO-DNA complex was collected through a 418 nm long pass interference filter and a 530 nm short pass dichroic filter. A 76-µm quartz flow tip was used during analysis and sorting on the EPICS 753.

All signals (integral, peak, and log integral fluorescence, and forward scatter) were processed through a gated amplifier triggered by fluorescence, to allow discrimination against low level nonspecific fluorescence. Objects were analyzed for logarithmic (LIFL) and linear integral fluorescence (IFL) and forward angle light scatter (FALS) relating to size. At least three histograms were generated: (1) LIFL versus FALS (64 × 64 channel); (2) frequency versus LIFL (256-channel, 3-decade log scale); and (3) frequency versus IFL (256- or 1024-channel). The high voltage (HV) of the photomultiplier (PMT) was adjusted so that the modes of the peaks of fluorescence corresponding to the G_2/M nuclei (or nuclei of the highest ploidy) would fall closer to the last channel of the 256-channel, 3-decade log histogram. The gain of the fluorescence amplifier or HV of PMT was adjusted so that the peaks corresponding to the largest chromosomes would fall closer to the last channel of the histogram of linear fluorescence. A gain setting greater than 5 gave signals that were not clearly discriminated. A gating region (bitmap) was set around the zone containing chromosomes in the LIFL versus FALS to eliminate extraneous signals from the conditioned histogram of fluorescence.

Flow sorting

Sorting was conducted on the EPICS 753 flow cytometer. Sheath fluid was ice-cold $MgSO_4$ buffer, without dithiothreitol, filtered through a 0.22-µm Millipore filter and kept on ice. Sorting windows were set on log or linear histogram of relative fluorescence intensity. About 2×10^4 objects from the selected peak area were collected directly onto a black nitrocellulose filter (Millipore Type AA, pore size 0.8 µm) placed on a filter paper cushion in a 60-mm Falcon plastic petri dish, in order to obtain high concentrations of objects over a small area.

Fluorescence microscopy

For identification of the sorted fractions, the piece of the filter that contained the sorted objects was carefully snipped out and soaked in dye solution (50 µg PI/ml, 100 µg MITH/ml, or 2.5 µg HO/ml) in $MgSO_4$ buffer for 5 min. The pieces of filter were mounted between a slide and a coverslip and observed under a Zeiss fluorescence microscope with an appropriate filter set. Photographs were taken with Kodak Ektachrome 400 ASA film or Kodak technical pan 2415 black and white film.

Bivariate flow cytometric chromosome analysis

For bivariate flow cytometric analysis, the chromosomes were stained with two stains, HO (1.25 µg/ml) and CA3 (12.5 µg/ml), about 1 h before the analysis. The chromosome suspensions were analyzed with an EPICS 753 flow cytometer equipped with two lasers. The first laser excited HO at 351–364 nm (100 mW) and the second laser excited CA3 at 458 nm (250 mW). Fluorescence resulting from the passage of chromosomes through each laser beam was collected through 405 and 495 nm long pass filters by a single photomultiplier tube. The two fluorescence signals measured on a single detector are separated in time by 7 µs, and are processed and acquired using the electronics provided with the EPICS 753. Both integrated fluorescence signals were acquired in bivariate histograms (Hoechst 33258 fluorescence versus chromomycin A3 fluorescence), with 64 or 128 channels on each axis. Chromosomes were analyzed at a rate of 150–300/s at all times.

Results

Obtaining high metaphase index

The metaphase index of the rapidly growing (36–48 h doubling time) tomato cells in suspension culture during

the log phase was less than 5%. Treatment with colchicine for an extended period increased the fraction of cells at metaphase in the population. The lowest effective colchicine concentration was 0.01%. The frequency of cells at metaphase increased in proportion to the colchicine concentration of up to 0.1% (data not shown). Figure 1 shows the metaphase index of the VFNT cherry tomato cells exposed to 0.1% colchicine for various time intervals. During the first 9 h of colchicine treatment, the frequency of cells at metaphase did not exceed 10–12%, but it increased steadily in the following hours with the peak at 18 h. Higher colchicine concentrations and longer exposure times produced lower metaphase indices. Up to about 45% metaphase index was obtained with VFNT cherry tomato cell suspension cultures treated with 0.1% colchicine exposure for 18 h; however, there were day-to-day variations in metaphase index of cells cultured under similar conditions. Metaphase indices between 20 and 35% were routinely obtained for most of the fast-growing tomato cell lines exposed to 0.1% colchicine for 18 h, 2 days after subculture. Sometimes, higher metaphase indices were obtained from 3-, 4-, or 5-day-old cultures, but results were more consistent with 2-day-old cultures.

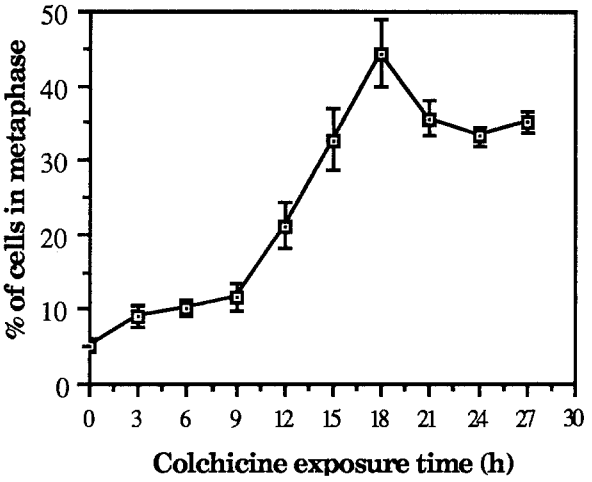

Fig. 1. Percentage of cells in metaphase in *L. esculentum* cv VFNT cherry cell culture after treatment with 0.1% colchicine. At least 250 cells were observed under the microscope to determine the metaphase index. Each point represents an average of four observations ± SE

Isolation of protoplasts

Since the cell wall is the main barrier for isolation of plant chromosomes, protoplasts were prepared by treatment of cells with wall-degrading enzymes. The enzymes used for this purpose also contain proteases and nucleases which could degrade nuclei and chromosomes. Thus, the conversion of cells to protoplasts was initially performed in a high osmolarity medium (Tan et al. 1987), which optimizes the release of intact protoplasts. Protoplasts were also washed with high osmolarity medium (~700 mOsm/kg of H_2O) to remove the enzymes before rupturing the plasma membrane, in order to release chromosomes and nuclei into a chromosome stabilizing medium.

In protoplasts isolated from mitotic cells in standard isolation medium, chromosomes either clumped together to form micronuclei or became decondensed (Fig. 2a). This made it impossible to prepare suspensions of monodispersed chromosomes. Tests of different osmolarities for protoplast isolation showed that high osmolarity was responsible for chromosome clumping (Table 1). Well-dispersed, unclumped metaphase chromosomes were observed in protoplasts isolated in culture medium without osmoticum but, in this case, the protoplasts were not intact. Table 1 shows the relationship between different osmolarities during protoplast isolation, clumping of chromosomes, and intactness of protoplasts. Intact protoplasts with little or no clumping of metaphase chromosomes could be obtained in a medium

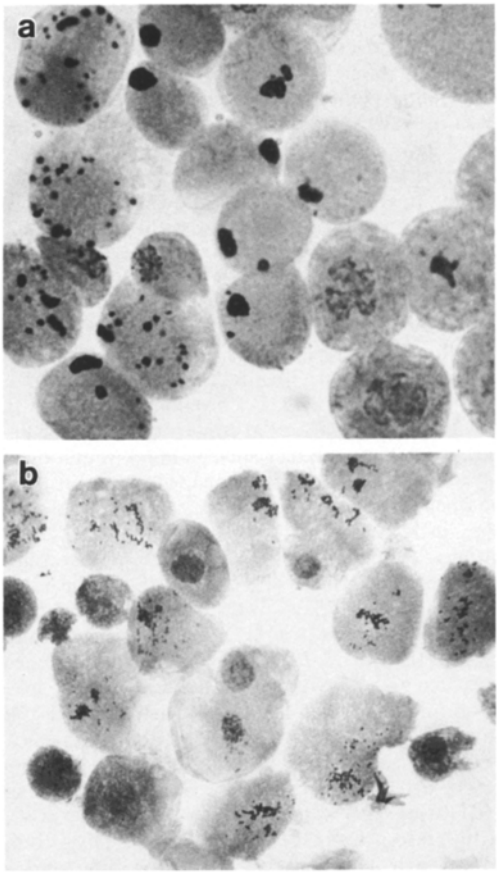

Fig. 2a and b. Protoplasts isolated from *L. esculentum* cv VFNT cherry cell suspension culture and stained with modified carbol fuchsin. **a** Protoplasts isolated in standard high osmolarity medium (711 mOs/kg H_2O) showing clumped chromosomes, micronuclei, and decondensed metaphase chromosomes. 630×. **b** Protoplasts isolated in low osmolarity medium (323 mOs/kg H_2O) showing dispersed, unclumped metaphase chromosomes. 250×

Table 1. Relationship between osmolarity of the protoplast isolation medium, clumping of metaphase chromosomes, and intactness of protoplasts. Protoplast isolation medium is tomato cell culture medium (without growth regulators) plus varying levels of KCl

KCl in protoplast isolation medium (m*M*)	Osmolarity (mOsm/kg H_2O)		Chromosome clumping	Protoplast intactness
	Without enzymes	With enzymes[a]		
300	799	907	+++	+++
240	641	806	+++	+++
180	543	697	+++	+++
120	431	582	++	+++
60	323	468	−	++
30	261	373	−	+
0	190	272	−	−

[a] 2% cellulysin and 1% macerase

with osmolarity of about 325 mOsm/kg of H_2O (Fig. 2b). Thus, for preparation of high-quality chromosome suspensions, the osmolarity of the protoplast isolation medium was adjusted to about 325 mOsm/kg of H_2O by adding KCl to a final concentration of 60 m*M* before the addition of wall-degrading enzymes.

Selective harvesting of mitotic protoplasts

A further enrichment of metaphase chromosomes was achieved by selectively harvesting mitotic cells from cell suspension cultures. Actively dividing cells were observed at the periphery of the microcalli in the suspension cultures. One-hour treatment of VFNT cherry cell suspension with cell wall digesting enzymes at 30 °C releases protoplasts from these cells, which are mostly in mitotic (M) or G_2 phase of the cell cycle. Undigested cell clumps composed mostly of nonmitotic cells were removed by passing the digestion mixture through a 80-μm nylon filter. Low speed centrifugation of the filtrate pelleted protoplasts and eliminated small particles. Up to about 80% of the protoplasts obtained from VFNT cherry cell suspensions using the procedure contain metaphase chromosomes. However, enrichment of mitotic cells from *L. pennellii* cell suspensions has not been attained by the above method. This may be due to the presence of smaller cell aggregates in the suspension cultures of *L. pennellii*.

Preparation of chromosome suspension

One of the most important steps in any chromosome isolation procedure is the swelling of the cells in hypotonic buffer (Trask 1989). In this step, the cells increase in volume and the chromosomes move apart from one another. Either $MgSO_4$ buffer (van den Engh et al. 1984) or a hypotonic solution of 75 m*M* KCl (Conia et al. 1987) caused swelling of tomato protoplasts, but the $MgSO_4$ buffer produced less cellular debris. After incubating with Triton X-100, protoplasts were physically ruptured by passing them forcefully through a Pasteur pipette to release the chromosomes into the chromosome stabilizing medium. Observation of chromosome suspensions prepared in $MgSO_4$ buffer and stained with DNA-binding stains under the fluorescence microscope showed monodispersed intact chromosomes with very little or no fluorescent debris. Rupturing protoplasts by syringing them through a hypodermic needle fitted on a syringe or by vortexing caused rupture of nuclei and breakage of chromosomes, and created a high level of DNA-containing debris which interfered with flow cytometric analysis of chromosomes.

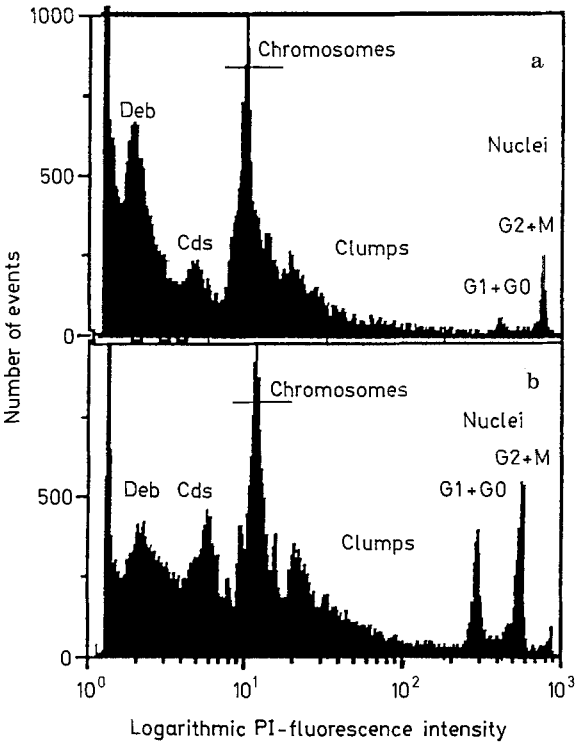

Fig. 3a and b. Univariate histogram of **a** *L. esculentum* cv VFNT cherry, and **b** *L. pennellii* obtained from PI-stained chromosome suspensions prepared in $MgSO_4$ buffer. About 40,000 events were analyzed using an EPICS Profile, with the photomultiplier tube voltage at 735. A logarithmic amplifier (3-decade, 256-channel) was used to amplify the signal to include all the events detected by the photomultiplier tube. The logarithmic transformation allows objects represented in equal numbers to have the same peak height and uniform peak width (Cram et al. 1989). Peaks of debris (Deb), chromatids (Cds), chromosomes, clumps of chromosomes, and nuclei were confirmed after examination of the flow-sorted objects under the microscope

Univariate flow cytometric analysis

Histograms of fluorescence distributions, such as the ones shown in Fig. 3, were routinely obtained from the

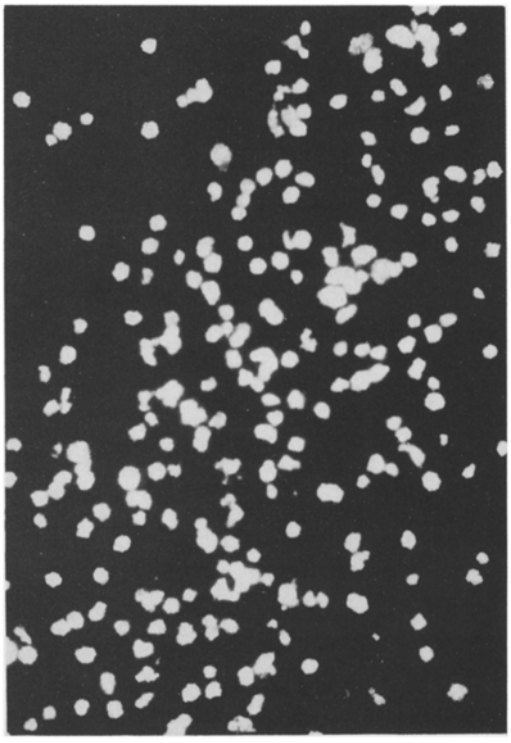

Fig. 4. Chromosomes of *L. esculentum* cv VFNT cherry collected onto a black nitrocellulose filter by flow-sorting and stained with PI. 1,150×. Chromosomes of different sizes are seen, representing 12 types of tomato chromosomes. Chromosomes morphology is not always preserved as some chromosomes decondensed to form micronuclei

analysis of tomato chromosome suspensions prepared in $MgSO_4$ buffer and stained with propidium iodide, Hoechst 33258, or mithramycin. The relative positions and surfaces of the various peaks and shoulders were reproducible in over 25 experiments. Figures 3a and 3b show histograms (for both *L. esculentum* and *L. pennellii*) of propidium iodide fluorescence intensities after logarithmic amplification, and include all the events detected by the photomultiplier tube. The 3-decade range of fluorescence intensities of this histogram can accommodate signals varying in amplitude by at least a factor of 1,000. Peaks corresponding to nuclei, chromosomes, chromatids, and low fluorescence cellular debris are clearly recognized by their relative fluorescence level. The nature of the peaks was confirmed by flow-sorting objects from each peak area on to filters and examining them with a fluorescence microscope at 400×.

The largest and most prominent peak (with minor peaks or shoulders) contained metaphase chromosomes (Fig. 4). Metaphase chromosomes corresponded to a large proportion (up to 48%) of the total objects in the chromosome suspensions prepared from VFNT cherry tomato suspension cultures in $MgSO_4$ buffer; nuclei represented less than 5% (Table 2). At least 10^5 metaphase

Table 2. Relative percentage of fluorescent debris, chromatids, chromosome, clumps, and nuclei in VFNT cherry tomato chromosome suspensions (PI-stained) prepared in different isolation media, as measured using a computer program provided on EPICS Profile. From 100 µl of each preparation, 60,000–80,000 events were analyzed

Medium (pH)	Debris (%)	Chromatids (%)	Chromosomes (%)	Clumps (%)	Nuclei (%)
$MgSO_4$ (8.0)	15.4	14.4	48.4	18.7	2.1
Polyamines (7.2)	18.7	14.4	44.5	22.2	3.2
Hexylene glycol (8.6)	36.5	12.6	31.2	17.2	2.5
KCl-PI (8.0)	32.6	19.6	35.7	11.1	0.9
Cell culture medium (5.8)	55.3	17.8	19.3	6.3	1.3

chromosomes can be collected by flow-sorting from chromosome suspensions prepared from one ml of VFNT cherry tomato cell suspension cultures.

Three peaks corresponding to nuclei were seen on the *L. pennellii* histogram in Fig. 3b. Their positions were equivalent to relative fluorescence intensities of 1:2:4. They represent G_0+G_1 nuclei (2), G_2+M nuclei (4C), and doublets of nuclei or different ploidy levels or a mixture of both. Nuclear peaks were less prominent in VFNT cherry (Fig. 3a) because the nuclei represented less than 5% of the total objects in the chromosome suspensions (Table 2). The peak of G_0+G_1 nuclei is practically nonexistent in VFNT cherry. This is mainly due to our procedure for selectively collecting mitotic cells and excluding most of the nonmitotic cells (G_0+G_1 nuclei) for chromosome preparation. This procedure is optimized only for the VFNT cherry cell line and not yet for the other cell lines. The area of the histogram between chromosomes and G_0+G_1 nuclei represents micronuclei and clumps of chromosomes.

A peak for chromatids was consistently seen and located at a position equivalent to one-half of the fluorescence intensity of the chromosome group. There were variations in the number of chromatids between cell lines. *L. pennellii* had a consistently higher level of chromatids than VFNT cherry.

Effect of different chromosome stabilizing buffers

Suspensions of intact tomato chromosomes were also prepared in various hypotonic buffers similar in content to the buffers used in isolation of chromosomes from mammalian cells (Gray and Langlois 1986) and plant

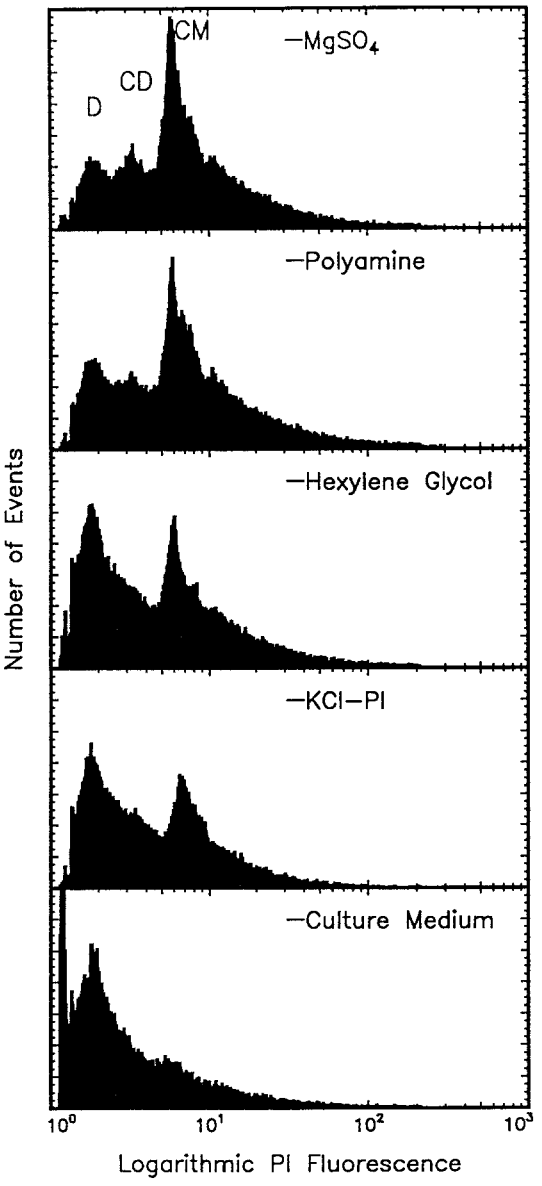

Fig. 5. Univariate histograms of *L. esculentum* cv VFNT cherry obtained from PI-stained chromosome suspensions prepared in $MgSO_4$ buffer, polyamine buffer, hexylene glycol buffer, KCl-PI buffer, and tomato cell culture medium. Regions D, CD, and CM were identified as debris, chromatids, and chromosomes respectively. From 100 µl of each preparation, 60,000–80,000 events were analyzed without changing instrument settings on EPICS Profile

cells (de Laat and Blaas 1984; Conia et al. 1987, 1989). Figure 5 compares the flow karyotypes obtained from chromosome suspensions prepared in $MgSO_4$ (van den Engh et al. 1984; Conia et al. 1989), polyamines (Sillar and Young 1981; de Laat and Blaas 1984), hexylene glycol (Wray and Stubblefield 1970; Hadlaczky et al. 1983), and KCl-propidium iodide (Buys et al. 1982) buffers and in tomato cell culture medium (Conia et al. 1987). Chromosome suspensions were stained with propidium iodide and analyzed on an EPICS Profile. The flow cytometer was first calibrated by analyzing a chromosome suspension prepared in $MgSO_4$ buffer; samples prepared in other chromosome isolation buffers were then analyzed without changing the instrument settings. $MgSO_4$ buffer and polyamine buffer produced a lower frequency of cellular debris and a higher frequency of intact chromosomes than hexylene glycol buffer or KCl-propidium iodide solution (Table 2). The chromosome suspensions prepared in tomato cell culture medium had a very high level of cellular debris, and the percentage of intact chromosomes among the total objects was low. $MgSO_4$ buffer is the best since it produces the least debris and the highest frequency of intact chromosomes and can also be used with most DNA stains. Other chromosome isolation buffers require addition of Mg^{++} ions for staining chromosomes with mithramycin or chromomycin A3.

Theoretical and observed univariate flow karyotype

It is possible to predict the univariate flow karyotype of an organism if the values of relative length or DNA content of the chromosomes are known. Conia et al. (1987, 1989) developed a formula for calculating univariate models of flow karyotypes of several plant species with computer software. They also prepared a theoretical flow karyotype (CV 2 and 3%) of tomato, using the data for chromosome length from Lapitan et al. (1989) (J. Conia, personal communication). Figure 6a–d shows theoretical univariate flow karyotypes of tomato prepared on a 256-channel linear scale, with coefficients of variation (CV) for each chromosome peak of 1, 2, 3, and 5%, respectively. The model assumes that there is no cellular debris and no noise due to the flow cytometer.

The theoretical flow karyotype shows that chromosomes 1 and 12 would form isolated peaks suitable for sorting. Chromosomes 2, 3, and 4 have similar lengths, so they would be identified as one composite peak, as would chromosomes 5, 7, 8, and 9. In the best case, with a CV of 2% (Fig. 6b), chromosome 6 on one side and chromosomes 10 and 11 on the other side would form shoulders on the latter peak. As CV increases, resolution of the peak decreases. The resolution of peaks, and thus the possibility of physically sorting chromosome types, is determined both by the inherent differences among the chromosomes and by the coefficient of variation, which in turn depends on the quality of chromosome preparation and on the resolution of the measurements.

Figure 7a and 7b shows observed linear fluorescence intensity distributions, due to tomato chromosomes and chromatids, in suspensions of *L. esculentum* and *L. pennellii*, respectively. Peaks are better resolved in *L. pennellii*. There are four main peaks for chromosomes (c1–c4). Chromosome peaks in the observed flow karyotypes resemble the theoretical distribution of tomato chromo-

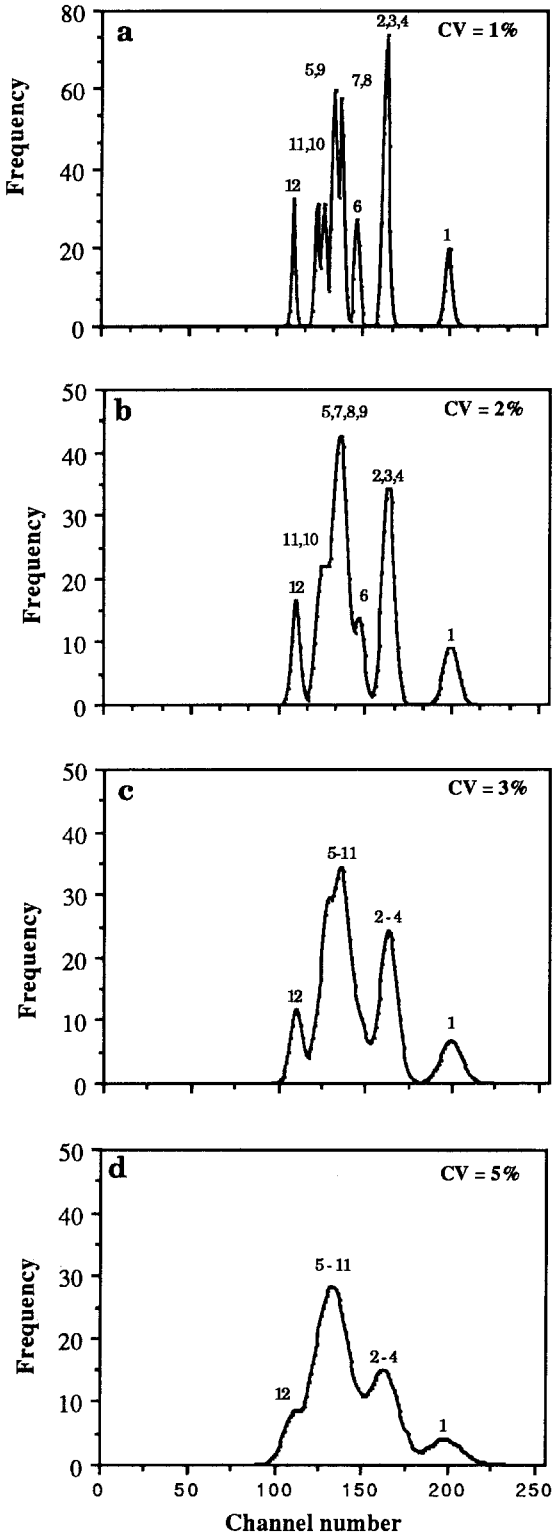

Fig. 6a–d. Theoretical univariate flow karyotypes of tomato chromosomes prepared according to Conia et al. (1989) using published values of their relative length (Lapitan et al. 1989). The simulations are based upon CV values of 1% (**a**), 2% (**b**), 3% (**c**), and 5% (**d**); the mode of the largest chromosome was set at channel 200. Peaks corresponding to the 12 tomato chromosomes are indicated. Resolution of the peaks decreases with increases in CV

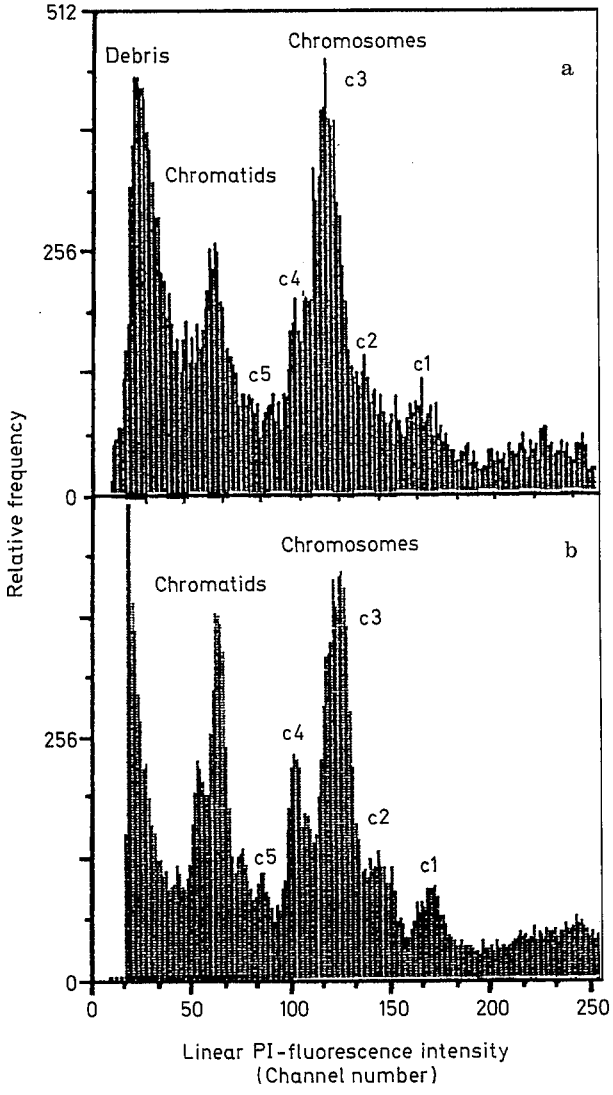

Fig. 7a and b. Univariate flow karyotypes obtained from PI-stained chromosome preparations (in $MgSO_4$ buffer) of *L. esculentum* cv VFNT cherry (**a**) and *L. pennellii* (**b**). Peaks corresponding to chromatids are also seen

somes when CV is 3% (Fig. 6c). Peaks for the chromatids reflect one-half the fluorescence intensities of the respective chromosome peaks. The peaks c1 and c5 may contain chromosome 1 and chromatids of chromosome 1, respectively. Chromosome 12 may be represented by peak c4. Peaks for the other chromosomes are not resolved clearly. Based on the theoretical karyotype, the largest peak, c3, may contain chromosomes 5, 6, 7, 8, and 9. Chromosomes 2, 3, and 4 may be in peak c2; however, the area of the peak c2 in the observed flow karyotype is smaller than expected to include all three of these chromosomes. We are now developing molecular techniques for determining the actual contents and purity of the sorted chromosomes represented in each peak.

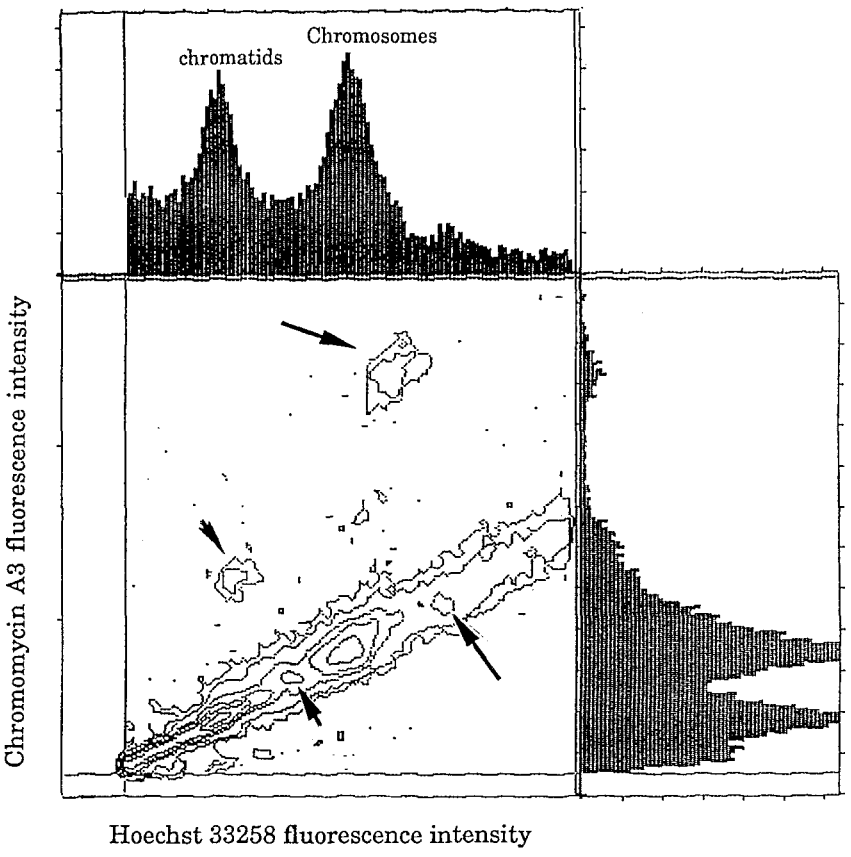

Fig. 8. Bivariate contour plot of CA3 fluorescence versus HO fluorescence and corresponding projections of the contour plot for chromosome suspensions prepared from *L. pennellii*. *Arrows* indicate well-resolved chromosomes (*large arrows*) and chromatids (*small arrows*)

Bivariate flow analysis of tomato chromosomes

Tomato chromosomes were analyzed using a double-stain combination, Hoechst 33258 and chromomycin A3. The bivariate histogram for *L. pennellii* is displayed as a contour plot in Fig. 8. The axis labelled Hoechst 33258 fluorescence corresponds to UV excitation, and the axis labelled chromomycin A3 fluorescence corresponds to 458-nm excitation. The fluorescences of AT-specific Hoechst 33258 and GC-specific chromomycin A3 are primarily determined by differences in base composition among chromosome types (Gray and Langlois 1986). Thus, in the bivariate karyotype, chromosomes may be separately resolved on the basis of differences in base composition and DNA content. The bivariate analysis shows two well-resolved peaks (indicated by large arrows). *L. pennellii* has at least one class of chromosomes with very high GC content (i.e., high chromomycin A3 fluorescence) in a well-resolved peak. Peaks corresponding to chromatids (indicated by small arrows) of these classes of chromosomes were also seen. The chromosomes and chromatids from these peaks can be flow-sorted and collected with high purity. We are now attempting to identify the chromosomes corresponding to these peaks.

Discussion

We have developed a reliable procedure for producing chromosome suspensions suitable for obtaining flow karyotypes of tomato. A key requirement for obtaining such chromosome suspensions is a high metaphase index in the cell population from which the chromosomes are to be isolated. Conia et al. (1987, 1989) took advantage of spontaneously occurring synchrony of the first division of the cultured leaf protoplasts of *Petunia hybrida* and *Nicotiana plumbaginifolia*, and combined it with a colchicine treatment to accumulate high frequency of metaphase cells. We could not use this procedure for tomato since the division frequency of tomato protoplasts has been very low. Cell suspension cultures of tomato, as in *Haplopappus gracilis* (de Laat and Blaas 1984), have proven to be a convenient source for isolation of chromosomes. Unlike *Haplopappus gracilis*, synchronization of tomato cell suspension culture with hydroxyurea (S-phase blocker) prior to colchicine treatment was not effective in increasing metaphase index (Roeder et al. 1989). As in *Papaver somniferum* (Hadlaczky et al. 1983) and *Petunia hybrida* (Conia et al. 1987), exposing fast-growing tomato cell suspensions to 0.1% colchicine, without hydroxyurea, accumulates a

sufficiently high frequency of metaphase cells. Tomato cells are partially synchronized to the G_0/G_1 portion of the cell cycle by growing the cultures to stationary phase. Then cultures are diluted with fresh medium so that the cells proceed through the cell cycle. Colchicine is added to arrest cells in metaphase when the largest portion of cells is about to divide. We were also able to enrich for mitotic cells by collecting protoplasts from the periphery of the cell clumps of VFNT cherry tomato. However, the enrichment of mitotic cells by this method may not be possible for cell suspension culture lines in which the cells are not in clumps (e.g., *L. pennellii*).

The extent of chromosome clumping and the fraction of DNA-containing debris fragments in the population determines the quality of the preparation. Minimization of chromosome clumps and DNA debris is important to flow karyotyping, since these often complicate quantitative analysis of the flow karyotype (Lozes 1989). They are also undesirable during sorting, since they reduce the effective sorting rate (by reducing the frequency of chromosomes in the population) and the purity of the sort (if they cannot be distinguished from the chromosomes to be sorted).

We have identified some factors that increase the quality of the chromosome suspensions. Preparing protoplasts in low osmolarity medium and swelling them in a hypotonic medium before lysis minimizes chromosome clumping. Rupturing protoplasts by passing them through a Pasteur pipette minimizes breakage of nuclei and chromosomes. Chromosome suspensions prepared in $MgSO_4$ buffer have minimal aggregation of cytoplasmic particles. Some cell lines consistently produce a higher level of debris than others. This is due to the presence of unhealthy or dead cells, which are susceptible to disruption during chromosome preparation. Unhealthy cells tend to have a high accumulation of starch grains (leucoplasts), which contribute to a high level of debris (unpublished observations). Production of populations enriched in healthy mitotic cells containing minimal DNA fragments requires culture conditions that maintain high viability, rapid cell division, and a high proportion of dividing cells. Thus, careful attention must be given to optimizing the culture of cells for preparation of chromosome suspensions to be used for sorting chromosomes with high purity and efficiency.

Chromosomes with single chromatids are observed mainly in the analysis of plant chromosome preparations (*L. esculentum*, *L. pennellii*, *Petunia hybrida*, and *Nicotiana plumbaginifolia*). Chromatids are not reported in the analysis of chromosome preparation from human or Chinese hamster cell lines. Chromatids may represent some of the contamination among the metaphase chromosomes and contribute to the underlying debris level. This can present a problem, since the peak(s) for smaller chromosomes would then also contain larger chromatids that would be sorted together. The peak corresponding to tomato chromosome 12 may overlap with the peak for chromatids of chromosome 1 since they have similar DNA content (Lapitan et al. 1989). Clumps of chromatids may also contaminate the peaks for metaphase chromosomes. Thus, the presence of chromatids is clearly not desirable for high resolution flow karyotyping and sorting of chromosomes with high purity. Long exposure to colchicine and mechanical forces during preparation and flow analysis has been suggested as the cause of metaphase chromosomes splitting into chromatids (Conia et al. 1987). Studies are in progress to minimize the production of chromatids.

Our ultimate goal is to discriminate each of the 12 tomato chromosome types by flow cytometry with sufficient resolution, so that they can be purified by sorting for use in gene mapping and cloning. The univariate flow karyotype of tomato obtained with propidium iodide should reflect interchromosomal DNA contents, since the chromosomal PI-fluorescence is unaffected by the differences in DNA base composition or sequence. Based on this flow karyotype, and according to the theoretical flow karyotype, large quantities of chromosomes 1 and 12 could be collected separately by flow-sorting with high purity. With a very high-resolution flow karyotype, chromosome 6 could also be collected, perhaps with less purity. Other chromosome types cannot be separated by univariate flow cytometry with propidium iodide. However, several fractions enriched for two or more chromosome types – (1) chromosomes 2, 3, and 4, (2) chromosomes 10 and 11, and (3) chromosomes 5, 7, 8, and 9 – could be collected from their composite peaks.

The bivariate flow karyotypes obtained with Hoechst 33258 and chromomycin A3 should reflect interchromosomal differences in AT contents or GC contents, respectively. The tomato genome has about 37% GC (Messeguer et al. 1991), but the short arm of chromosome 2 has about 23 Mbp of tandemly repeated 45S ribosomal RNA genes with more than 50% GC content (Kiss et al. 1988; Perry and Palukaitis 1990). The well-resolved peak with high GC content on the bivariate karyotype (Fig. 8) may therefore contain chromosome 2. We are now exploring various methods for the identification of chromosome types or their enrichment in flow-sorted fractions. These include: (1) fluorescence *in situ* hybridization, (2) polymerase chain reaction (PCR) using oligonucleotide primers for chromosome-specific markers, and (3) spot-blot hybridization analysis (Lebo 1989; Minoshima et al. 1990). Preliminary results from spot-blot hybridization of chromosomes sorted from the two well-resolved peaks in the bivariate flow karyotype (Fig. 8) identify the peak with high GC content as chromosome 2 and the other as chromosome 1 (G. Martin, unpublished results).

Acknowledgements. We thank Dr. P. Jackson for providing the opportunity for K.A. to train at the National Flow Cytometry facilities at Los Alamos National Laboratory, and Dr. J. Conia for helpful discussions during the course of this work and for providing the theoretical flow karyotype of tomato (2 and 3% CV). This work was supported by a grant from the Cornell Biotechnology Program (which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, the U.S. Army Research Office, and the National Science Foundation), and by the U.S. Department of Energy.

References

Buys C, Koerts T, Aten J (1982) Well identifiable human chromosomes isolated from mitotic fibroblasts by a new method. Hum Genet 61:157–159

Conia J, Bergounioux C, Perennes C, Muller P, Brown S, Gadal P (1987) Flow cytometric analysis and sorting of plant chromosomes from *Petunia hybrida* protoplasts. Cytometry 8:500–508

Conia J, Bergounioux C, Brown S, Perennes C, Gadal P (1988) Caryotype en flux biparamétrique de *Petunia hybrida*. Tri du chromosome numéro I. CR Acad Sci Paris 307:609–615

Conia J, Muller P, Brown S, Bergounioux C, Gadal P (1989) Monoparametric models of flow cytometric karyotypes with spreadsheet software. Theor Appl Genet 77:295–303

Cram LS, Bartholdi MF, Ray FA, Cassidy M, Kraemer PM (1989) Univariate flow karyotype analysis. In: Grey JW (ed) Flow cytogenetics. Academic Press, London New York, pp 113–136

DuPont FM, Staraci LC, Chou B, Thomas BR, Williams BG, Mudd JB (1985) Effect of chilling upon cell cultures of tomato. Plant Physiol 77:64–68

Engh G van den, Trask B, Cram S, Bartholdi M (1984) Preparation of chromosome suspensions for flow cytometry. Cytometry 5:108–117

Gray JW, Langlois RG (1986) Chromosome classification and purification using flow cytometry and sorting. Annu Rev Biophys Biophys Chem 15:195–235

Griesbach RJ, Malmberg RL, Carlson PS (1982) An improved technique for the isolation of higher plant chromosomes. Plant Sci Lett 24:55–60

Hadlaczky G, Bisztray G, Praznovszky T, Dudits D (1983) Mass isolation of plant chromosomes and nuclei. Plant 157:278–285

Kao KN (1982) Staining methods for protoplasts and cells. In: Wetter LR, Constabel F (eds) Plant tissue culture methods. National Research Council, Saskatoon, Canada, pp 67–71

Kiss T, Kis M, Abel S, Solymosy F (1988) Nucleotide sequence of the 17S–25S spacer region from tomato rDNA. Nucleic Acids Res 16:7179

Laat AMM de, Blaas J (1984) Flow-cytometric characterization and sorting of plant chromosomes. Theor Appl Genet 67:463–467

Lapitan NLV, Ganal MW, Tanksley SD (1989) Generation of tomato somatic chromosome karyotype based on *in situ* hybridization of TGR I satellite repeat. Genome 32:992–998

Lebo RV (1989) Gene mapping strategies and bivariate flow cytogenetics. In: Grey JW (ed) Flow cytogenetics. Academic Press, London New York, pp 225–242

Lozes C (1989) Cell culture for chromosome isolation. In: Grey JW (ed) Flow cytogenetics. Academic Press, London New York, pp 35–42

Malmberg RL, Griesbach RJ (1980) The isolation of mitotic and meiotic chromosomes from plant protoplasts. Plant Sci Lett 17:141–147

Matthews BF (1983) Isolation of mitotic chromosomes from partially synchronized carrot (*D. carota*) cell suspension cultures. Plant Sci Lett 31:165–172

Messeguer R, Ganal MW, Steffens JC, Tanksley SD (1991) Characterization of the level, target sites, and inheritance of cytosine methylation of tomato nuclear DNA. Plant Mol Biol (in press)

Minoshima S, Kawasaki K, Fukuyama R, Maekawa M, Kudoh J, Simizu N (1990) Isolation of giant DNA fragments from flow-sorted human chromosomes. Cytometry 11:538–546

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nitsch JP (1969) Experimental androgenesis in *Nicotiana*. Phytomorphology 19:389–404

Perry KL, Palukaitis P (1990) Transcription of tomato ribosomal DNA and the organization of the intergeneric spacer. Mol Gen Genet 221:102–112

Roeder M, Arumuganathan K, Lapitan NL, Tanksley SD, Earle ED (1989) A rapid method for large-scale isolation of tomato metaphase chromosomes. In: Galling G (ed) Proc Braunschweig Sym Appl Mol Biol, pp 137–140

Sillar R, Young BD (1981) A new method for the preparation of metaphase chromosomes for flow analysis. J Histochem Cytochem 281:74–78

Tan MMC, Rietveld EM, Marrewijk GAM van, Kool AD (1987) Regeneration of leaf mesophyll protoplasts of tomato cultivars (*L. esculentum*): factors important for efficient protoplast culture and plant regeneration. Plant Cell Rep 6:172–175

Trask B (1989) Chromosome isolation. In: Grey JW (ed) Flow cytogenetics. Academic Press, London New York, pp 43–60

Van Dilla MA, Deaven LL (1990) Construction of gene libraries for each human chromosome. Cytometry 11:208–218

Wray W, Stubblefield E (1970) A new method for the rapid isolation of chromosomes, mitotic apparatus, or nuclei from mammalian fibroblasts at near neutral pH. Exp Cell Res 59:469–478