Chapter 39

Human Chromosome Analysis and Sorting

Judith A. Fantes and Daryll K. Green

1. Introduction

Flow cytometry has provided the cytogeneticist with a fast and accurate method of measuring the quantity of DNA in each human chromosome (*1*). Almost all the chromosomes in the human complement can now be resolved and abnormal chromosomes and aneuploidies (13, 21, and X) recognized. A flow karyotype shows a pattern of peaks and troughs that is unique for each individual or cell line because of the variation in heterochromatic regions of the chromosomes (2). When combined with family studies, flow cytometry has been able to resolve homologues differing in DNA content by as little as 1/2000 of the human genome (3, 4), less than a metaphase band. In addition, the sorting capabilities of most flow machines have provided a method for the purification of small but useful quantities of individual chromosomes, for example, 2x 10^6 average sized human chromosomes are equivalent to 500 ng of DNA. Using recombinant DNA techniques, this material can be used to generate a large number of DNA probes to produce a chromosome-specific library, which can be

used for the molecular analysis of genetic disease (*5,6*). More recently, molecular biologists have experimented with gene mapping by sorting small quantities of individual chromosomes onto filters for spot-blot hybridization with DNA probes (*7*).

The sample preparation and flow machine techniques relating to all the above biological objectives will be discussed here. The art of producing an enriched sample of a particular group of human chromosomes by flow cytometry lies in bringing a clean, well-separated chromosome suspension to a clean, sterile, and well-adjusted flow machine. Debris, unbalanced stain/chromosome concentration or clumps of aggregated chromosomes in the suspension or noise in the flow machine in the form of obstruction to the flow, optical misalignment, or electronic noise all contribute to a reduction in purity of the sorted sample. The methods described here are aimed at producing the best possible sample obtainable from a starting material of peripheral blood lymphocytes or lymphoblastoid cells to the end point of verifying the identity and purity of the chosen sorted chromosome group.

2. Materials

1. Complete culture medium: RPMI 1640, 10% fetal calf serum, 12.5 m*M* MOPS, 100U/mL penicillin, 100 µg/mL streptomycin.
2. Phyto-hemagglutinin (PHA): reagent grade from Wellcome.
3. Lymphoprep from Nyegaard.
4. Polyamine buffer (B1): 15 m*M* Tris, 0.2 mM spermine, 0.5 m*M* spermidine, 2 m*M* EDTA, 0.5 m*M* EGTA, 80 m*M* KCl, 20 m*M* NaCl, 14 m*M* (0.1% v/v) β-mercaptoethanol. Adjust to pH 7.2 with 1*N* HCl before adding β-mercaptoethanol. Prepare fresh every week; store at 4°C.
5. Polyamine buffer plus digitonin (B2): 0.1% solution of digitonin in B1. Prepare a saturated solution by heating to 37°C and filtering through a 0.2 µm filter to remove any undissolved digitonin. The best source of digitonin is Fluka, since some batches of digitonin from other sources are difficult to dissolve.
6. Hoechst 33258: 100 µ*M* in distilled water. Store in dark at 4°C.
7. Ethidium bromide: 1 mg/mL in distilled water. Store in dark at 4°C.
8. Chromomycin A3: 1 mg/mL in distilled water. Leave overnight in cold to dissolve. Store in dark at 4°C.
9. Colcemid: 0.01 mg/mL in distilled water. Filter sterilize and store at 4°C.

10. DAPI (4,6-Diamidino-2-phenylindole•2HCl): 50 µg/mL in distilled water. Store in dark at 4°C.
11. Spermidine *bis*-acridine (*8*): Dissolve 5 mg in 2 mL methanol. Make up to 100 mL with 10 m*M* disodium orthophosphate, adjust to pH 6.5 with 0.2*M* HCl. Store in dark at 4°C.
12. Decon or 7X detergent diluted 1:25 with sterile filtered distilled water.
13. Activated glutaraldehyde (Cidex).
14. Distilled water sterilized by filtration through 0.2 µm filter.
15. Phosphate buffered saline (Dulbecco A); filter sterilized.

3. Methods

The method of isolating chromosomes described here uses polyamines to stabilize the chromosomes and detergent treatment to lyse the cells (*9*). Since the lysis of interphase nuclei is minimal, this method can be used successfully with all types of cell lines, including suspension cultures with a significant proportion of interphase cells. Although the isolated chromosomes are highly condensed, they maintain most of their in vivo structure, and it is possible to prepare high mol wt DNA from them.

3.1. Cell Culture

Human chromosomes are usually prepared from lymphoblastoid cell lines (EBV transformed B lymphocytes, *see* Chapter 5, this volume) or from PHA-stimulated peripheral blood lymphocytes. The best chromosome suspensions and hence the flow karyotypes with the greatest resolution are prepared from cultures with a reasonable proportion of mitotic cells (>20%, *see* Note 1).

1. Lymphoblastoid cells: set up cells from a stationary phase culture at 3×10^5 viable cells/mL. Thirty hours later, add colcemid to a final concentration of 0.1 µg/mL to block the cells at metaphase. Sixteen to eighteen hours later harvest cultures and disperse any cell clumps by gentle pipeting.
2. Lymphocytes: Defibrinate a 40-mL sample of peripheral blood with orange sticks. Spin down the cells at 400*g* for 10 min, and remove serum (≈20 mL). Replace with an equal volume of medium without FCS. Layer 10 mL of suspension onto 7 mL lymphoprep in a sterile plastic centrifuge tube. Spin for 15 min at 800*g*. The lymphocytes collect at the interface and can be removed with a pastette. They are washed

twice by centrifugation with excess complete medium and counted before setting up at 0.5×10^6 cells/mL in complete medium containing 15% fetal calf serum and 1% PHA. Colcemid is added to 0.1 μg/mL after 46 or 66 h of incubation, and the cultures are harvested 17 h later.

3.2. Chromosome Preparation

1. Take an aliquot of cell suspension for a cell count. Centrifuge cells at 180*g* for 10 min. Pour off supernatant and resuspend cells in fresh ice-cold complete medium. Centrifuge cells at 180*g* for 10 min; this washing step removes some dead cells and debris.
2. Pour off the supernatant and resuspend cells in hypotonic 0.075*M* KCl solution to swell the cells. Use 10 mL of hypotonic for every 10^7 cells.
3. Incubate the lymphoblastoid cells for 20 min at 37°C; peripheral blood lymphocytes require 10 min incubation at room temperature at this stage.
4. Remove 0.25 mL for mitotic index determination: add 5 mL of 3:1 methanol:acetic acid and allow to stand for 10 min. Centrifuge at 300*g* for 5 min, pour off the supernatant, and resuspend the pellet in a small volume of fixative (*see* Note 2). Drop onto a clean slide, and dry quickly in air. Stain in 2% Giemsa in pH 6.8 buffer and air dry. Count the number of divisions in 500 cells.
5. After incubation in hypotonic solution, centrifuge the cell suspension at 180*g* for 5 min. All further steps should be carried out at 4°C.
6. Pour off the supernatant and resuspend the pellet in cold polyamine buffer (B1), 1 mL/10^7 cells. Centrifuge at 180*g* for 5 min.
7. Pour off the supernatant. Resuspend the pellet in cold polyamine buffer plus digitonin (B2), 1 mL/10^7 cells (*see* Note 3).
8. Vortex vigorously for 30–60 s to break the cell walls. Monitor cell lysis by phase-contrast microscopy or by fluorescence microscopy. In this case, drop the chromosome suspension onto a slide previously spread with a drop of fluorochrome such as Hoechst 33258 or ethidium bromide. Place a coverslip in position, seal with rubber solution, and examine. Most of the chromosomes should be free and in suspension after 60 s vortexing; further vortexing will only cause an increase in chromosome degradation and stickiness.
9. Nuclei should be removed from the chromosome suspension before flow analysis and sorting, since they can contaminate sorted fractions. Spin down the nuclei at 180*g* for 10 min, and transfer the

supernatant carefully to another tube. Add 1 mL of B2 buffer to the pellet and resuspend by a 5s vortex. Centrifuge at 180*g* for 5 min, remove supernatant, and add to the first supernatant. Check for the presence of nuclei as described above in stage 8 (*see* Note 4).

10. This chromosome suspension can be stored for 2–4 wk at 4°C with little loss of resolution when analyzed by flow cytometry.

3.3. Staining

1. If the chromosome suspension was prepared from cells with a high mitotic index (> 30%), dilute 1:1 with fresh B2 buffer before staining (*see* Note 5).
2. For single fluorochrome analysis, add Hoechst 33258 to 0.5 μg/mL or ethidium bromide to 50 μg/mL.
3. For dual fluorochrome analysis (*see* Note 6), add chromomycin A3 (*see* Note 7) to 20 μg/mL, magnesium chloride to 1 m*M* and Hoechst 33258 to 0.5 μg/mL from stock solutions. Leave for at least 1 h for the fluorochromes to equilibrate before analyzing or sorting the chromosomes.

3.4. Chromosome Identification

Although chromosomes prepared in polyamine buffer (*see* Notes 8 and 9) after exposure to 16 h colcemid are condensed, it is possible to band and identify them if they are swollen and elongated slightly by prior exposure to phosphate buffered saline. Not all the chromosomes on a slide will be sufficiently decondensed to give adequate banding for identification, but over 50% should have sufficient bands and examples are shown in Fig. 1.

1. Sort 60,000 chromosomes into a cold Eppendorf tube containing 0.25 mL of buffer B2. On our machine, this quantity of chromosomes will be sorted in 0.25 mL of PBS sheath fluid so the sorted chromosomes will finally be exposed to 1:1 buffer B2:PBS.
2. Fix chromosomes by adding 40% formaldehyde to give a final concentration of 4%. Leave for 10 min on ice.
3. Spin chromosomes onto alcohol-cleaned slides using a Shandon cytocentrifuge at 150*g* for 7 min. 60,000 chromosomes can be split between two slides.
4. Allow slides to air dry. Wash briefly in deionized water and air dry.
5. Fix in 3:1 methanol:acetic acid for 5 min and air dry.

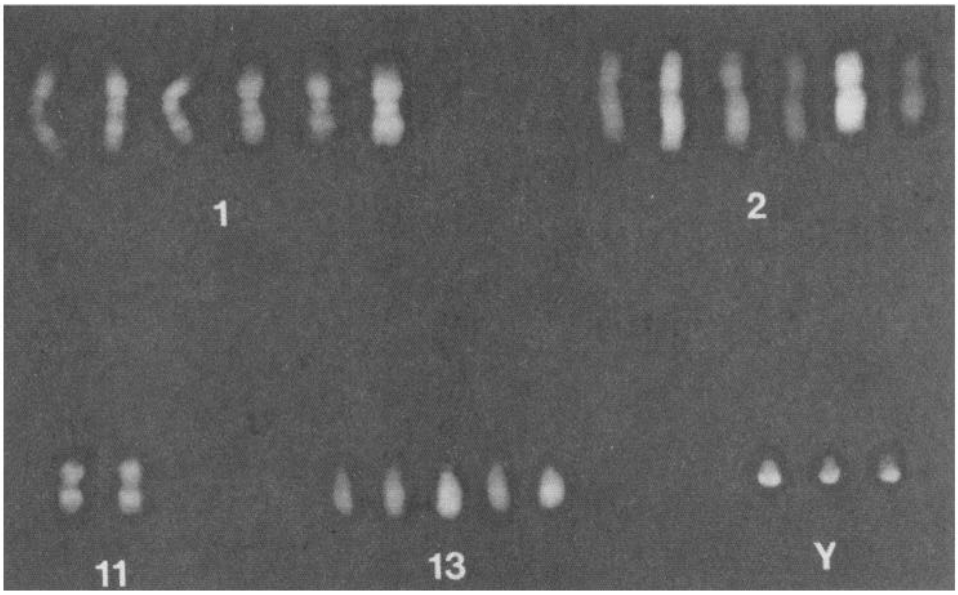

Fig. 1. Selected photomicrographs of sorted chromosomes 1,2,11,13 and Y. The chromosomes were sorted by flow cytometry, and deposited on microscope slides according to the method described in the text. Dapi staining was used for chromosomes 1,2,11, and 13 and spermidine *bis*-acridine for chromosome Y. The distinctive banding of the chromosomes associated with these dyes can be seen.

6. Either: (a) Stain in 0.5 µg/mL DAPI in distilled water for 10 min, wash, and air dry. Mount in citifluor/glycerol; or (b) Stain in 0.005% spermidine *bis*-acridine for 10 min, wash, and air dry. Mount in deionized water.

3.5. Flow Cytometry

The following methods do not relate to any particular commercial flow cytometer, but given that a machine consists of a light source (usually a laser beam), an optical train, a liquid flow arrangement to deliver the chromosome suspension, and a signal detection system, they are generally applicable to all machines. A general text dealing with the basic principles and a wide variety of applications of flow cytometry is recommended for a newcomer to the subject (*10*). A typical bivariate flow karyotype of normal human chromosomes is shown in Fig. 2.

The analysis of human chromosomes requires the flow cytometer to be performing as well as or better than specification. It is not good enough to have the instrument roughly tuned to performance, which often suf-

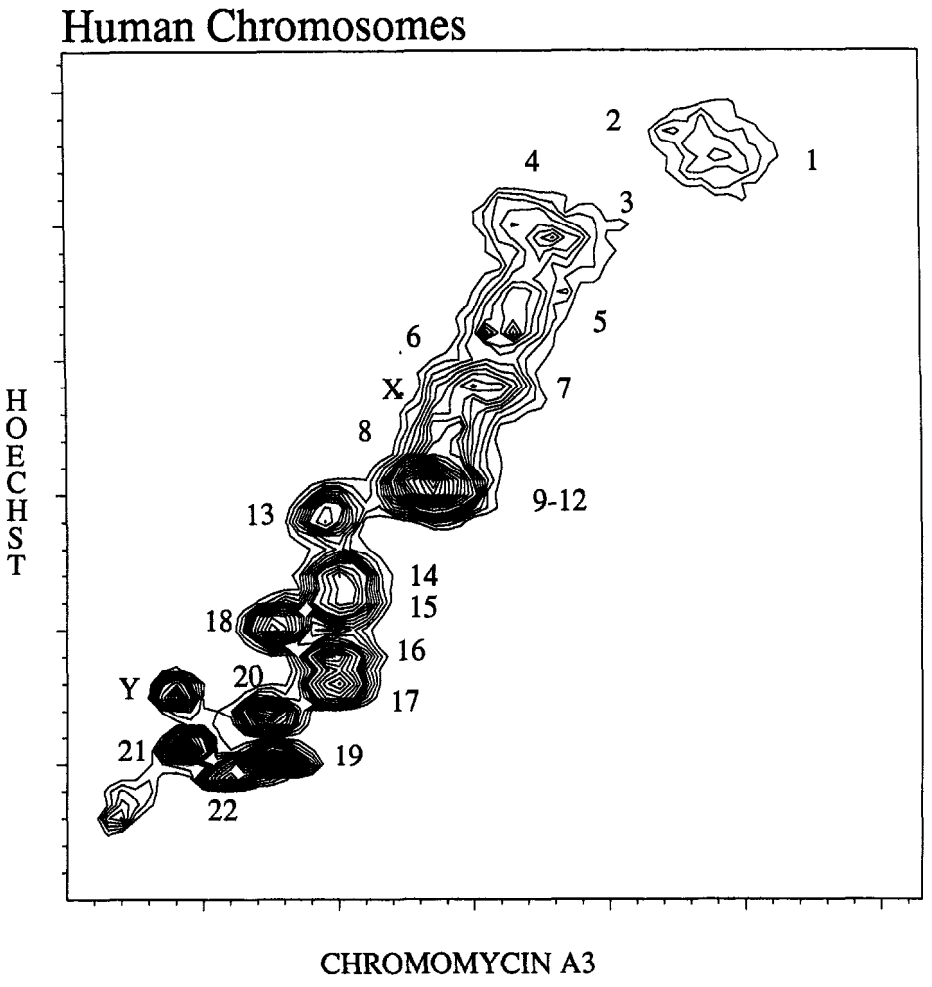

Fig. 2. Human chromosome fluorescence distribution for two DNA-specific fluorescent dyes Hoechst 33258 and Chromomycin A3. Differential amounts of AT- and GC-rich DNA on each chromosome, which determines the intensity of Hoechst and Chromomycin fluorescence, respectively, produce further separation of the chromosome peaks than is seen with a single DNA-specific fluorochrome.

fices for cell analysis, and attention to detail will be rewarded with a reduced coefficient of variation (CV) of the chromosome peaks, provided of course that the suspension of chromosomes is well prepared. It will be assumed that the reader has some knowledge of a particular flow cytometry machine, and is able to adjust the signal detection and display system, clean all lens and signal detection surfaces, and optimize the output of the lasers or other light sources. Those parts of the system that are most likely to give rise to performance loss when they are not optimized are dealt with in detail below.

3.5.1. Sample Delivery

The arrangement of nozzles and pressurized sheath and sample stream is designed to deliver chromosomes in single file through a well-defined position in the cytometer, where a beam of light can be focused. All tubes, nozzles, and reservoirs therefore must be clean, aligned, and purged before injecting a series of samples. This will ensure an unobstructed laminar flow, which leads to precise positioning of chromosomes and a stable droplet formation. Any liquids introduced into the cytometer must be filtered through a 0.2 μm filter.

1. Cleaning (*see* Note 10): Flush the following solutions through the machine for 30 min each. This should include backflushing through the sample stream.
   a. Warm Dilute 7-X (or equivalent) solution to remove chromosome material from previous experiments.
   b. Cidex to sterilize.
   c. Use distilled water to rinse out unwanted detergent or Cidex.
   d. Sheath buffer chosen for the experiment.
2. Alignment: In many cases, the sample and sheath nozzles have a fixed geometry, but in cases where adjustment is provided, make sure that the sample injection nozzle is placed centrally inside the sheath nozzle and terminates where the inside diameter of the sheath nozzle is widest. This can be checked by injecting a concentrated fluorescent dye (preferably the dye to be used in subsequent experiments) through the sample stream and focusing the laser beam somewhere near the signal detection point such that fluorescent light piping occurs right up to the sample nozzle tip. Figure 3 shows an example of sheath and sample nozzles both in and out of correct adjustment. Take care with plastic sheath nozzles not to melt the plastic when looking for a light piping effect.
3. Purging: Every flow cytometer is equipped with a liquid exhaust port well above the sample injection nozzle, which can be opened to release trapped air bubbles. These bubbles must be exhausted before a stable flow and droplet formation can be established.
4. Sample pressure: Too high a sample pressure can cause the sample stream to balloon, as shown in Fig. 3, and leads to a broad final sample stream and consequently a high chromosome peak CV. Most commercial flow cytometers are equipped with a simple pressurized sample delivery system, which is adequate but sometimes oversensitive to adjustment when low sample to sheath pressure differentials

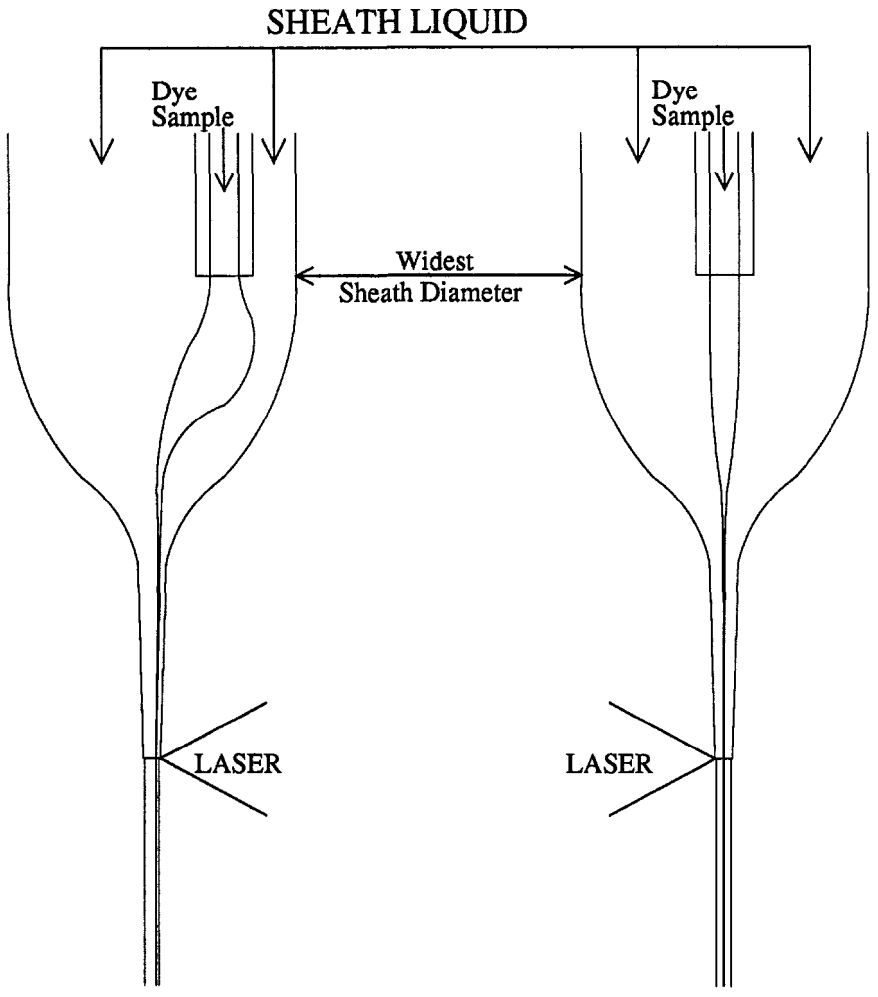

Fig. 3. The example shown is for a glass nozzle system for "in air" flow where the laser beam is focused on the edge of the sheath nozzle to produce light piping in the concentrated dye sample. On the left, the sample nozzle is shown misaligned, and a high sample pressure is also shown to be causing ballooning of the emerging sample stream. On the right, the dye sample emerges at an acceptable relative pressure and passes centrally down to the "in air" detection point. Alignment and sample pressure are equally important for cuvette detection systems.

are needed. A more satisfactory way of controlling sample flow is to use a motorized syringe driver capable of delivering less than 1 mL from a 1-mL syringe. A cooling jacket around the syringe will maintain the chromosomes in good condition throughout an experiment.

5. Sample stream coating: A stable flow karyotype will be achieved more rapidly when, prior to the introduction of a chromosome sus-

pension, a "dummy" sample of buffer and dye (at twice the final concentration) is injected through the sample stream for approximately 5 min. Using a "dummy" sample between different chromosome suspensions also helps to flush out remaining chromosomes from previous samples.

Generally, flow cytometers have a sheath liquid pressure of about one atmosphere. Under these conditions, a chromosome suspension containing 10^7chromosomes/mL flowing at 0.4 mL/h, which leads to a flow rate of about 1000 chromosomes/s, should result in a well-resolved flow karyotype. A slower sample flow rate may produce even better resolution, and depending on the quality of cytometer adjustment and the intensity of the laser beam(s), a faster flow rate may not necessarily spoil the resolution. As high a flow rate as possible should be achieved for chromosome sorting experiments.

3.5.2. Beam Alignment

1. Single beam experiments: It is important that the laser beam passes precisely along the optical axis of the focusing lens system. Check this by first removing the forward light scatter detector and then adjust the laser beam to pass through an aperture at the geometric center of the final focusing lens, and at the same time check that the beam is incident on the center of a screen some distance away from the stream axis. Swing the liquid stream to one side for this adjustment. A mark, known to be on the optical axis, on an adjacent wall often provides a useful target for beam alignment checking. Swing back the liquid stream and adjust the beam focus at the stream to be at its narrowest by observation through the stream viewing optics.
2. Dual beam experiments: Here, the lower wavelength beam, which in our example is the ultraviolet beam, is aligned in a similar way to the single beam experiments. The higher wavelength beam is aligned sufficiently off axis to allow the spherical and chromatic aberrations to compensate and bring the two beams into focus along the liquid stream axis. Figure 4 shows how the longer focal length of the higher wavelength beam and the shorter focal length of off axis rays produce the desired alignment of focal points.

3.5.3. Fluorescence Detection

To optimize the detection of each fluorescent color, it is important to select suitable long and short pass filters. The following combinations

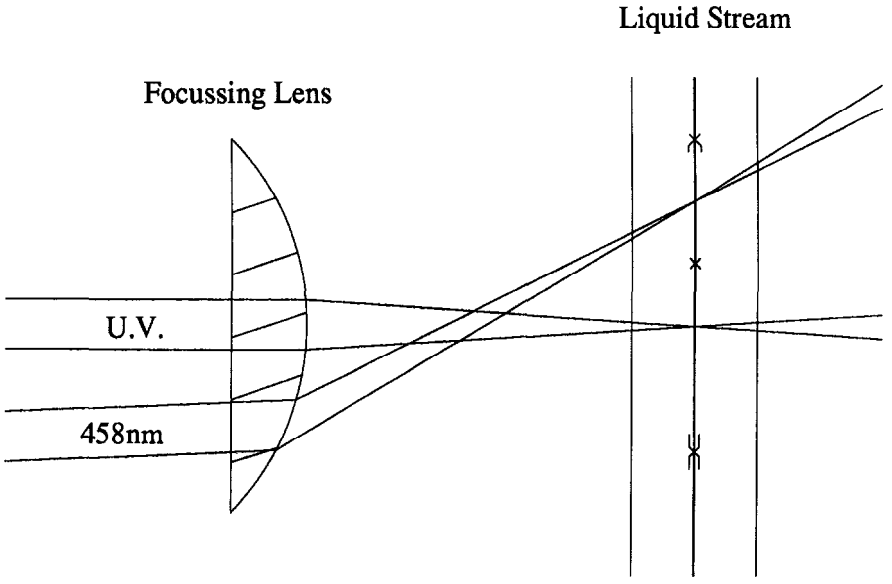

Fig. 4. The focusing arrangement of an ultraviolet (U.V.) and a blue 458 n*M* wavelength beam is shown. A converging lens that could be cylindrical or convex focuses the U.V. beam on axis and the 458 n*M* beam at a slight angle and off axis. The scale of distances and beam widths are somewhat exaggerated in the figure, as are the chromosomes in the liquid stream.

were used for the specific fluorescent dyes described here: (a) Hoechst 33258–475 n*M* long pass + 550 n*M* short pass, (b) Chromomycin A3–515 n*M* long pass.

3.5.4. Final Adjustment

The final adjustment of laser beam, stream position, flow rate, and photomultiplier detector positioning needs an actual sample of flowing objects. Standard practice is to use a suspension of fluorescent microbeads. This is not recommended prior to chromosome analyses and sorting, since there is a risk of chromosome aggregation around stray microbeads left behind after the tuning process. Use instead a portion of the chromosome suspension. The distribution of signal pulses appearing on the oscilloscope will soon become familiar, particularly the prominent cluster of signals, all with the same peak height, arising from the human chromosome groups 9 to 12. Optimize the peak height and minimize the peak width of the signal pulses by adjusting the optical components and stream position in an ordered fashion. Readjustment of the stream position between adjustment of each optical component will usually ensure a steadily improving signal height and width.

3.5.5. Sorting

A working day will, as a rule, produce 10^6 sorted chromosomes. Reaching this target depends on a clean chromosome suspension, a steady flow rate of 1000 to 2000 chromosomes/s, and a degree of stability of the "live parts" of the flow machine. The purity of each sorted fraction will depend on the resolution of chromosome peaks seen in the flow karyotype and on prior identification of the chromosome or chromosomes appearing in particular peaks of interest. Identification requires a sorted fraction of about 60000 chromosomes into 0.25 mL of sheath buffer followed by the procedure described in the chromosome identification section. Contamination of a sorted fraction with chromosomes from adjacent peaks can be minimized to a limited extent by drawing narrow sort windows, but the researcher must be sure that the extra purity gained is justified by a possible further day's sort to accumulate the required quantity of chromosomes. It is usually possible to be occupied on another task while each sample of chromosome suspension is sorted, but it must be possible to check frequently on the relative positioning of sorting windows, chromosome peaks, and the droplet stability. No amount of care and attention to the machine will improve sorted fraction purity if there are large numbers of undividing nuclei or large quantities of debris in the original suspension.

4. Notes

1. The starting point for a good chromosome preparation must be a rapidly dividing cell culture with very few dead cells and free from bacterial or mycoplasma contamination. Mycoplasma contamination will cause the chromosomes in the final suspension to stick together and form large clumps.
2. It is important to treat the cells gently during the preparation; centrifuge at low speeds, resuspend the cell pellet by tapping the tube not by vortexing, and ensure that all the cells are uniformly exposed to hypotonic and buffer solutions by maintaining a single-cell suspension.
3. The concentration of cells to buffer B2 in methods, stage 7 is important; if the amount of B2 is decreased, cell breakage will be incomplete.
4. The differential centrifugation step described in stage 9 will remove most of the contaminating nuclei. Centrifugation of the chromosomes at higher speeds to concentrate them or remove debris will increase the number of clumps and degraded chromosomes giving

poor resolution. It is better to start again from a culture with a higher mitotic index.

5. Do not attempt to analyze very concentrated chromosome suspensions. Staining irregularities will occur as well as excessive signal coincidences and nozzle blockages. Dilute with buffer B2.
6. Addition of 10 m*M* sodium citrate and 10 m*M* sodium sulfite (*11*) to stained chromosomes has been reported to increase the resolution of dual beam flow karyotypes.
7. Chromomycin A3 intercalates into the DNA, and this has sometimes reduced the efficiency of subsequent DNA manipulations. This problem has been overcome by dialysis of the sorted chromosomes against two changes of B1 to remove the stain.
8. Chromosomes stabilized by polyamines are not suitable for chromosome-mediated gene transfer into other cells. An alternative buffer containing 15 m*M* Tris, 3 m*M* calcium chloride, and digitonin should be used (*12*).
9. Chromosomes stabilized with polyamines are also not suitable for use with antibodies against chromosomal proteins; other buffers are recommended (*13*).
10. We have found that the best routine for cleaning the tubes of the flow machine involves washing first with warm detergent solution to remove residual chromosomes without fixing them to the tube walls, and then sterilizing with activated glutaraldehyde (Cidex). Extensive washing with sterile distilled water is then necessary to remove all traces of Cidex. Using 70% ethanol, which is commonly used by flow cytometer operators for flushing and sterilizing, will fix residual chromosomes to the tube walls. The method recommended here avoids this problem.

References

1. Langlois, R. G., Yu, L. C., Gray, J. W., and Carrano, A. V. (1982) Quantitative karyotyping of human chromosomes by dual beam flow cytometry. *Proc. Natl. Acad. Sci. USA* **79**, 7876–7880.
2. Green, D. K., Fantes, J. A., Buckton, K. E., Elder, J. K., Malloy, P., Carothers, A., and Evans, H. J. (1984) Karyotyping and identification of human chromosome polymorphisms by single fluorochrome flow cytometry. *Hum. Genet.* **66**, 143–146.
3. Harris, P., Boyd, E., Young, B. D., and Ferguson-Smith, M. A. (1986) Determination of the DNA content of human chromosomes by flow cytometry. *Cytogenet. Cell Genet.* **41**, 14–21.

4. Harris, P., Cooke, A., Boyd, E., Young, B. D., and Ferguson-Smith, M. A. (1987) The potential of family flow karyotyping for the detection of chromosome abnormalities. *Hum. Genet.* **76,** 129–133.
5. Krumlauf, R., Jeanpierre, M., and Young, B. D. (1982) Construction and characterisation of genomic libraries from specific human chromosomes. *Proc. Natl. Acad. Sci. USA* **79,** 2971–2975.
6. Cooke, H. J., Fantes, J. A., and Green, D. K. (1983) Structure and evolution of human Y chromosome DNA. *Differentiation* **23,** S48–S55.
7. Lebo, R. V., Gorin, F., Fletterick, R. J., Kao, F., Cheung, M., Bruce, B. D., and Kan, Y. W. (1984) High resolution chromosome sorting and DNA spot-blot analysis assign McArdle's syndrome to chromosome 11. *Science* **225,** 57–59.
8. Van de Sande, V. H., Lin, C. C., and Dengau, K. V. (1979) Clearly differentiated and stable chromosome bands produced by a spermine bis-acridine; a bifunctional analogue of quinacrine. *Exp. Cell Res.* **120,** 439–444.
9. Sillars, R. and Young, B. D. (1981) A new method for the preparation of metaphase chromosomes for flow analysis. *J. Histochem. Cytochem.* **29,** 74–78.
10. Melamed, M. R., Mullaney, P. F., and Mendelsohn, M. L. (eds.) (1979) *Flow Cytometry and Sorting* (Wiley, New York).
11. Van den Engh, G., Trask, B., and Gray, J. W. (1987) Improved resolution of bivariate flow karyotypes by manipulation of staining conditions. *Cytometry* **Suppl. 1,** 3.
12. Porteous, D. J., Morten, J. E. N., Cranston, G., Fletcher, J. M., Mitchell, A., van Heyningen, V., Fantes, J. A., Boyd, P. A., and Hastie, N. D. (1986) Molecular and physical arrangements of human DNA in HRAS1-selected, chromosome mediated transfectants. *Mol. Cellular Biol.* **6,** 2223–2232.
13. Trask, B., Van den Engh, G., Gray, J. W., Vanderlaan, M., and Turner, B. (1984) Immunofluorescent detection of histone 2B on metaphase chromosomes using flow cytometry. *Chromosoma* **90,** 295–302.