Materials and Methods

The Preparation of Human Chromosomes for Flow Cytometry

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Conventional cytogenetic analysis of human chromosomes based on the examination of metaphase spreads is a time-consuming process relying on the skill of the cytogeneticist. It is possible, however, to use flow cytometry to examine monodispersed preparations of human chromosomes that have been stained with two DNA-binding dyes of differing base-pair specificities (Hoechst 33258, which has an adenine/thymidine binding preference and Chromomycin A3, which has a guanine/cytosine binding preference). Several good reviews (Metezeau *et al.*, 1993; Monard & Young, 1994) give details of the development of both human and animal flow cytogenetics and the most common uses to which flow cytometry is put. These can be summarised as:

- looking for chromosome deletions, translocations and marker chromosomes

- sorting chromosomes either in large numbers (1-2 × 10^6) for genome analysis or small numbers for the production of PCR-generated chromosome 'paints'.

The production of a suspension of chromosomes for flow analysis is a time-consuming and skilled process and the analysis and sorting of chromosomes so produced requires a good knowledge of the set-up and operation of the flow sorter.

Cells

It should be possible to use any human cell to produce a chromosome preparation. Both suspension and monolayer cell lines are suitable, as are phytohaemagglutinin-stimulated peripheral white blood cells. Whichever cell type is used, optimal preparations will be made from healthy, exponentially growing cells.

Reagents

Colcemid solution
Cell culture medium appropriate to the cell type
Hypotonic solution (75mM KCl)
Chromosome isolation buffer (CIB):

(pH 7.2	20mM NaCl
in autoclaved	80mM KCl
distilled water)	15mM Tris-HCl
	0.5mM EGTA
	2mM EDTA
	0.15% w/v 2-mercaptoethanol
	0.2mM spermine (free base)
	0.5mM spermidine (free base)

Digitonin
Propidium iodide (50µg/ml in 0.1% Triton-X100)
Hoechst 33258 (100µg/ml in distilled water)
Chromomycin A3 (2mg/ml in ethanol)

Method

1. Subculture cells 24 hours before blocking with 0.05µg/ml colcemid for 5-16 hours depending on the rate of cell growth. If using monolayer cells, harvest using trypsinisation.

2. Centrifuge all harvested cells at 100*g* for 10 minutes. Resuspend in fresh medium. At this point, estimate the proportion of cells in mitosis by taking an aliquot, pelleting and adding 0.5ml propidium iodide and running on a benchtop flow cytometer. Spin again at 100*g* for 10 minutes.

3. Discard supernatant by inverting the tube, making sure that all surplus liquid is removed. Disaggregate the pellet gently by flicking the tube. Add 5ml of hypotonic solution, mix and leave for 10-30 minutes at room temperature. This causes the cells to swell and the process can be monitored microscopically. When the cells are swollen but not disrupted, spin at 100*g* for 10 minutes.

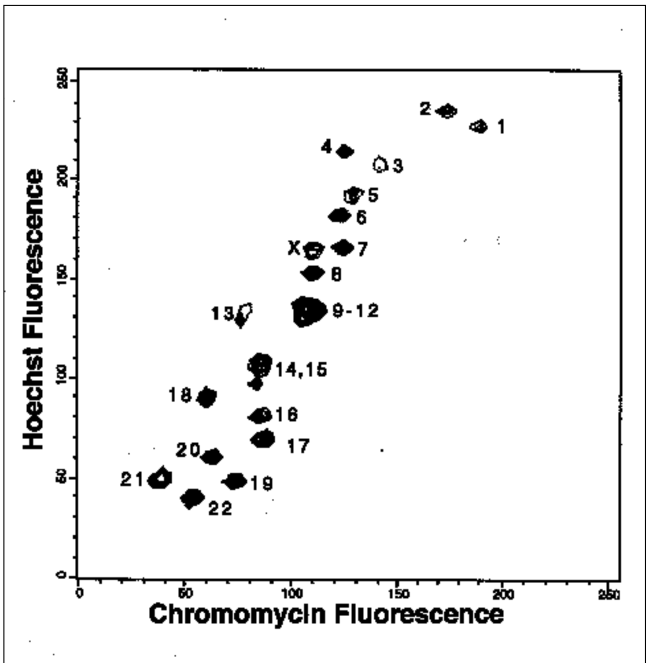

Fig. 1. Flow karyotype from a normal human female. All the chromosomes, with the exception of the 9-12 group, can be resolved.

4. Dissolve 12mg of digitonin in 5ml of distilled water (this needs to be done on a hot plate). Allow to cool and add 1ml of CIB. Make up the volume to 10ml with distilled water and adjust the pH to 7.2. Place on ice.

5. Remove supernatant from cells using a pipette and agitate gently to disrupt pellet. Add 10 times the volume of cold digitonin/CIB and mix gently. This causes the cells to disrupt and release the chromosomes.

6. Mix a small amount of the preparation with propidium iodide and view under a fluorescence microscope. If the chromosomes are not released from the metaphase nuclei, vortex gently.

7. The chromosome preparation may be kept in the refrigerator for several weeks.

Chromosome staining

Transfer 1ml of the chromosome preparation to a tube. Add 30µl Hoechst 33258, 40µl 15mM $MgCl_2$ and 50µl chromomycin A3, mix and leave in the dark at 4°C for 2 hours. The profile may be improved by adding 100µl sodium citrate (100mM) and 100µl sodium sulphite (250mM) 15 minutes prior to running on the cytometer.

Cytometer set up

A dual laser cell sorter should be used. A typical set-up would be as follows:
Primary laser set to UV, approximately 300mW to excite the Hoechst dye, secondary laser at 457.9nm, approximately 300mW to excite chromomycin. The trigger parameter should be the Hoechst fluorescence.

Collect Hoechst emitted fluorescence at 390-480nm and Chromomycin emitted fluorescence above 500nm. The intensity of signal from each chromosome type will be dependent on the total fluorescence, i.e. chromosome size, with the larger chromosomes appearing to the top right, smaller ones in the bottom left. The differences in base-pair ratio mean that the different chromosome types appear at varying degrees off the diagonal. In this way all types, with the exception of the 9-12 group, can be identified (Figure 1). Variations such as chromosome deletions, replications and translocations can often be detected by examination of the flow karyotype (Figure 2).

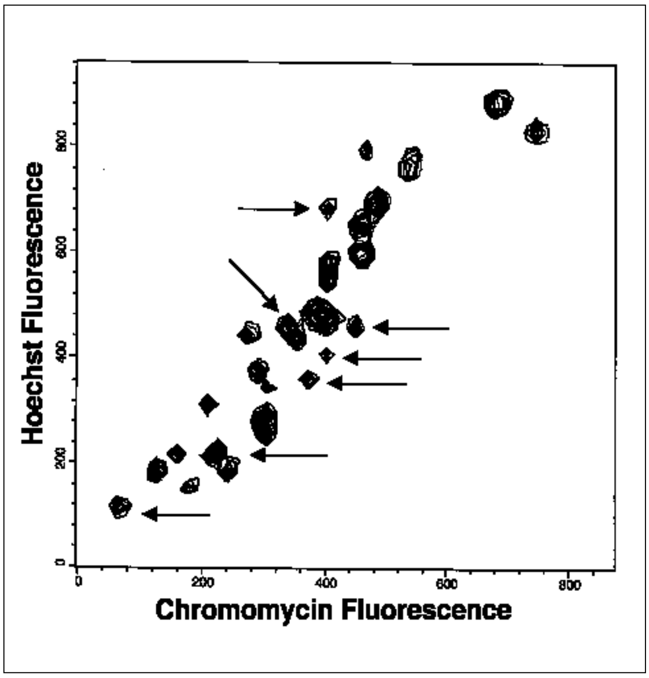

Fig. 2. Flow karyotype from a primary bladder carcinoma showing several derivative chromosomes (arrowed).

References

Metezeau, P., Schmitz, A. & Frelat, G. (1993) Analysis and sorting of chromosomes by flow cytometry: new trends. *Biology of the Cell* **78**, 31-39.

Monard, S.P. & Young, B.D. (1994) Chromosome and analysis and sorting by flow cytometry. In *Flow Cytometry: A Practical Approach* (3rd Edn.) (Ed. M.G. Ormerod). IRL Press.