STUDIES OF CHROMOSOMES AND NUCLEI

USING FLOW CYTOMETRY

PROEFSCHRIFT

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To my parents and to Ger

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Chapter 1

GENERAL INTRODUCTION

The total length of the DNA helix present in each mammalian cell has been calculated to be two meters. With the help of special proteins, the DNA is packed into a sphere 5-10 μm in diameter: the nucleus. The complex of DNA and protein is called chromatin. Shortly before a cell divides, the DNA becomes even more tightly coiled and is divided into a number of separate, compact packages, which can be observed under the microscope. These are called chromosomes. The number and size of the chromosomes are well-defined for each mammalian species. Human mitotic cells contain 46 chromosomes ranging in size from 2 to 10 μm (figure 1.1).

Chromosomes carry the genetic information of a cell. The cell reads this information in very much the same way as one reads a printed message. The sequence of the nucleotide bases, which make up the DNA molecule, codes for the proteins that control function and form. Each cell of the body contains a complete copy of the DNA and, therefore, a complete set of genetic instructions. The stretches of DNA that code for single hereditary characteristics are called genes. One chromosome may contain thousands of genes. At a given moment, only a small fraction of the genetic material is being translated. The location of the genes on the chromosomes and in relation to regulating sequences is important for their expression in the active genome. Changes in the sequence of the DNA, such as deletions or translocations, may have serious consequences for cell function, and ultimately for the health of the organism. Occasionally, changes in the DNA sequence, and in the protein for which it codes, can be advantageous. The diversity of life forms testifies to the role of these changes during evolution.

One aspect of the organization of chromosomes of many species can be made visible under the microscope. Special staining techniques produce a striped pattern along the length of the chromosomes. These bands can be seen at high magnification through a microscope (figure 1.1). Each chromosome has a characteristic banding pattern. The pattern provides a basis for the identification of the chromosomes. Figure 1.2 shows the banding pattern, or idiotype, of chromosomes from a normal human cell.

Figure 1.1. A metaphase spread of a human mitotic cell, showing the differences in size and banding patterns among the 46 chromosomes (figure compliments of A. Hagemeijer, Rotterdam, The Netherlands).

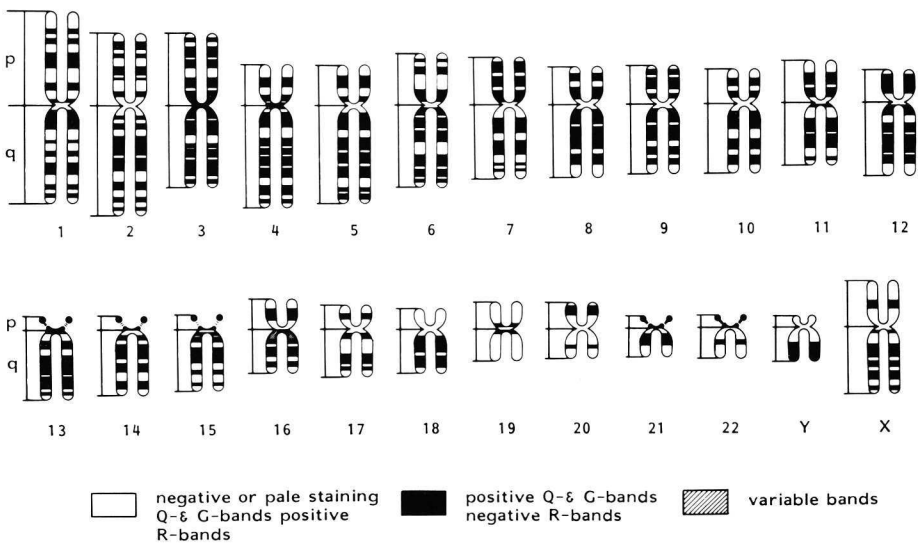


Figure 1.2. The idiotype of human chromosomes showing the pattern of the chromosome bands (reprinted with permission from McDermott, 1975).

These staining procedures, and more elaborate techniques capable of producing more than 6000 bands in the human genome, have made it possible to detect the structural rearrangements in chromosomes that are reflected in abnormal banding patterns. Chromosomal rearrangements and variations in chromosome number (aneuploidy) have been associated with various cancers and inheritable human disorders. The Philadelphia chromosome (t9;22) in chronic myelocytic leukemia, a consistent translocation (t8;14) in Burkitt's lymphoma, and trisomy of chromosome 21 in Down's syndrome are prominent examples. Radiation, chemical mutagens and viruses have also been shown to produce lesions in chromosomes.

Various techniques have made it possible to progress from the study of banding patterns to the study of the molecular organization of chromatin. At a structural level, X-ray diffraction, electron microscopy and antibody labelling are a few of the techniques being used to unravel the 3-dimensional organization of histones, non-histone proteins, and DNA in chromatin and chromosomes.

Our knowledge of the organization of chromosomes at the level of the nucleotide sequence itself is increasing at a rapid rate. The sequence, location, and regulation of genes, as well as the protein products of genes, can be studied with the introduction of techniques grouped under the term recombinant DNA technology. These include restriction enzyme digestion, DNA cloning and nucleic acid hybridization. DNA fragments can be multiplied (cloned) by inserting them into plasmids or bacterial viruses, which are then grown in bacteria. An endless variety of DNA sequence probes can be produced in pure quantities in this way. Conditions can be created in the laboratory under which the proteins are produced that are encoded in the DNA sequence of these fragments. The cloned DNA sequences can also be used to construct a map of the genome in cells and chromosomes.

Using restriction enzymes, the organization of genes in the genome can also be studied. DNA is cut by these enzymes at very specific sites. The resulting DNA fragments are separated by gel electrophoresis. Gene sequence probes are matched by Southern blot hybridization to the highly specific restriction fragment patterns in these gels (figure 1.3). For molecular hybridization, the DNA is denatured, or separated into single strands. Under the proper conditions, DNA sequence probes will reanneal to form double-stranded complexes where the nucleotide sequence of the probe and the DNA in the gel match well, or are complementary. If the probe is radioactively labeled, the position of the hybridized probe is visualized by autoradiography. Probes that overlap a splicing point help to link the fragments together in the construction of a complete sequence. In these

restriction maps, the molecular organization in normal and abnormal cells or in different species can be compared.

Because nucleic acid sequences occupy precise positions in cells and chromosomes, a great deal of information is lost when these molecules are extracted from cells by homogenization. Exciting new techniques make it possible to determine the location of specific DNA sequences in chromosomes. In this way, a link can be made between the genomic map and classical cytogenetics. Because chromosomes form a discrete subdivision of the total genome, they can be physically separated. Gene sequences probes can be hybridized in Southern blots or on filters to the DNA isolated from suspensions enriched for individual chromosome types. This is a rapid means of assigning genes to the chromosomes on which they reside.

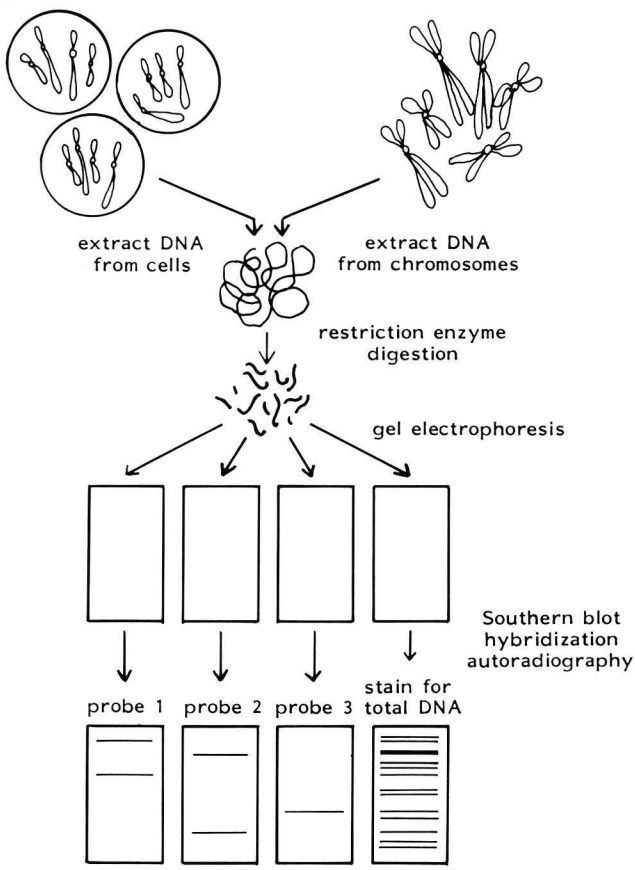


Figure 1.3. Principles of Southern blotting for the construction of the genomic map.

DNA sequences can be more precisely located in chromosomes using a technique called in situ hybridization. In this technique, the nucleic acid sequence probes are hybridized to chromosomes fixed to slides, after the slide has been exposed to conditions which separate the base pairs in the DNA helix. The specific locations of the bound probe on the arms of the chromosomes can be visualized using radioactive, fluorescent, or enzymatic labeling procedures (figure 1.4). The location of the sequences can also be determined in altered chromosomes present in abnormal cells.

Alternatively, purified fractions of a given chromosome can be used as the starting material for the cloning of DNA fragments using recombinant DNA techniques. A set of cloned DNA fragments is called a library. One can obtain, for instance, a library from the X or Y chromosome. Pre-enrichment of a given chromosome before cloning greatly simplifies the search for clones producing DNA sequences unique to that chromosome. "Walking" along the genome, or using overlapping DNA sequence probes to sequentially identify neighboring sequences, is easier when DNA from a single chromosome type has been cloned. Chromosome-specific probes are also useful tools in in situ hybridization studies for the identification of chromosomes, even when they are translocated to a new position, and for the enumeration of chromosomes. Recombinant clones prepared from defective chromosomes can also be a source of probes. These probes can be used to study the defect at a molecular level or to study the disorder's consequences for gene products and cell function.

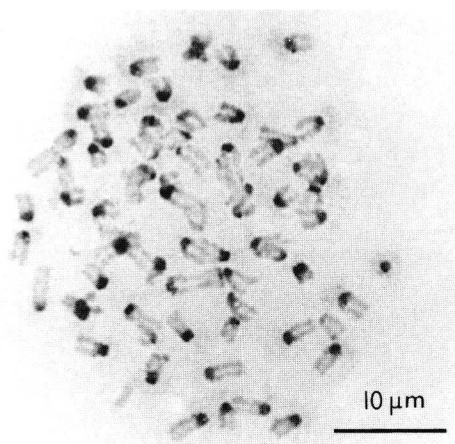


Figure 1.4. A metaphase spread of a mitotic cell from a transformed mouse in vitro culture showing the localization of mouse satellite DNA sequences to the acrocentric ends of the chromosomes. The chromosomes were labeled using an immuno-enzymatic in situ hybridization technique (Landegent et al., 1984).

The paragraphs above illustrate that there are now many techniques with which chromatin and the genome can be studied. The techniques fall into two general classes: 1) those in which the properties of a population of cells or chromosomes, as a whole, can be characterized, and 2) those that allow the analysis of individual cells or chromosomes, but require their microscopical observation. When the properties of entire populations are averaged, as in the first group of techniques, information about any heterogeneity among the cells or chromosomes is lost. On the other hand, the analysis of individual cells or chromosomes with a microscope is still slow. Without microscope-based microfluorimeters, the observations are difficult to quantify, making them often subjective.

In recent years, instruments have been developed that make it possible to rapidly quantify the optical properties of individual cells, as well as to separate and purify selected cells. This technique, flow cytometry, has also been extended to the analysis of chromosomes. If the molecular properties of chromosomes and nuclei can be successfully translated into

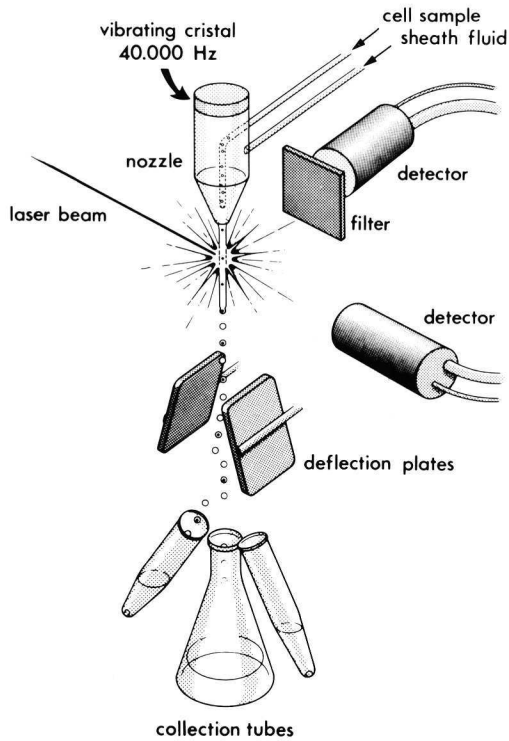


Figure 1.5. The basic features of a flow cytometer with sorting capabilities.

optical properties, this technique offers a combination of features that have limited other techniques in the analysis of the genome.

For flow cytometric analysis, cells or cell fragments are suspended in solution and are stained with fluorescent dyes. In the flow cytometer, cells are forced in a narrow stream through the path of an intense light source, such as a laser (figure 1.5). The particles pass the laser beam in single file at a rate of several thousand per second. When the cells enter the light spot, they scatter light or emit fluorescence. As each particle passes through the laser, its optical properties are quantified and stored. Large numbers of cells can be measured individually, and the optical properties of a cell population can be determined in a short time. Many flow cytometers also have the ability to sort cells. For this purpose, the sample stream is broken into droplets after the point where the optical measurements are performed. Droplets containing desired cells are given an electric charge and are deflected into a collection tube by the influence of an electrostatic field.

Perhaps the best known application of flow cytometry is in the measurement of the DNA content of individual cells. During the cell cycle (figure 1.6), an accurate copy of the DNA in a cell is made. When a cell divides, the two daughter cells each receive one copy of the DNA. If a population of cells is stained for DNA content using a DNA-specific fluorochrome, the fluorescence intensity (and, thus, the DNA content) of each particle can be measured in a flow cytometer. Fluorescence

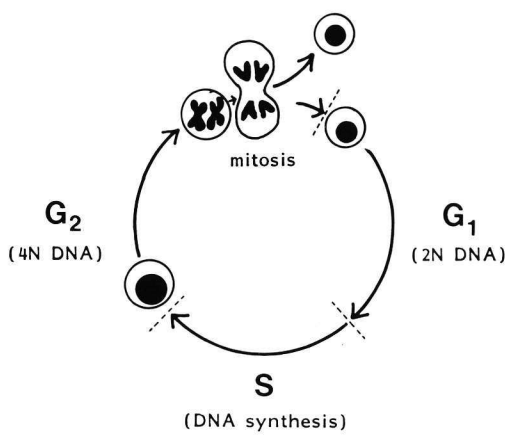


Figure 1.6. The cell cycle of mammalian cells. Cells in G_1 have the normal diploid content of DNA. During S phase, DNA is synthesized to double this amount. G_2 denotes the cell cycle phase when cells have twice the normal DNA content (4N). Just before mitosis, the chromosomes become visible. One copy of each chromosome goes to each daughter cell when the cell divides at mitosis.

distributions, such as the one shown in figure 1.7, can be measured. The amount of DNA in each cell indicates its position in the cell cycle. Therefore, the distribution of cells among the cell cycle phases can be assessed from the form of the DNA fluorescence distribution. The presence of cells containing an abnormal amount of DNA (which may be due to an extra chromosome or to a chromosome of abnormal size) will also be reflected in the DNA distribution.

At mitosis, the DNA in a nucleus is partitioned over the chromosomes. If the chromosomes are released from mitotic cells, stabilized in solution and stained with fluorescent dyes, they too can be measured in a flow cytometer. Because chromosomes have only a fraction of the DNA content of whole nuclei, the measurement requires equipment with a high degree of sensitivity and precision. Figure 1.8 shows a DNA fluorescence distribution of the chromosomes of cultured Chinese hamster cells. The peaks represent individual chromosomes. For this species, all the chromosomes are resolved. The relative peak positions in the distribution contains information about the set of chromosomes in the cells. It is for this reason that the fluorescence distribution is termed a flow karyotype. Human cells contain many more, and smaller, chromosomes than Chinese hamster cells. Using the best chromosome isolation and staining procedures

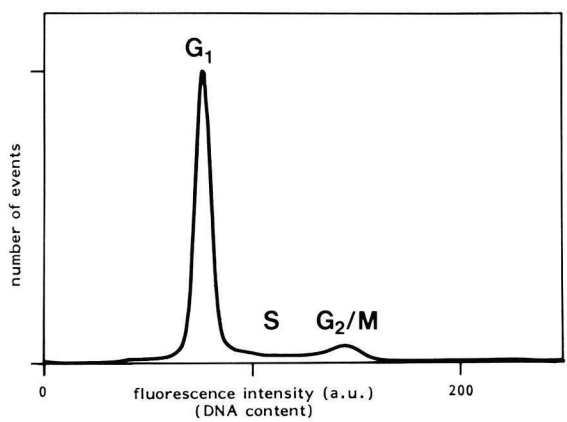


Figure 1.7. Fluorescence distribution of a population of fixed cells stained for DNA content with propidium iodide. The fluorescence intensity measured is proportional to the DNA content of each cell. The tall peak represents the cells in G_1 with 2N DNA. Cells in G_2 or M have twice the DNA content and form the small peak to the right. The fluorescence intensities between the two peaks represent cells with an intermediate amount of DNA, or those in S phase. The area under the peaks gives an approximation of the relative number of cells in G_1 or G_2 + M in the cell population being analyzed. This population consists of cells in G_1.

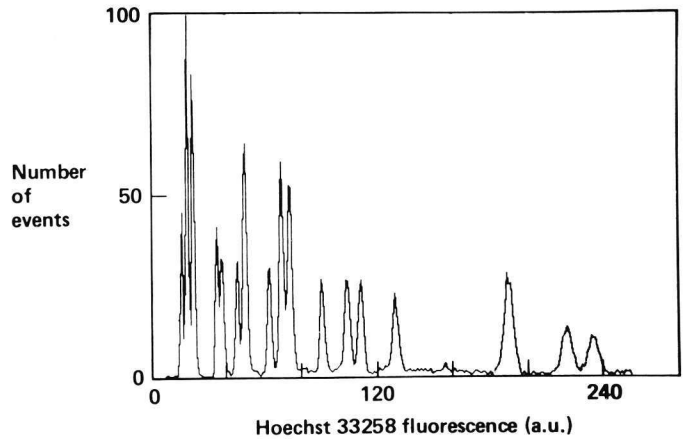


Figure 1.8. A flow karyotype of Chinese hamster chromosomes. The chromosomes were isolated from mitotic cells as described in chapter 6. The DNA in the chromosomes was stained with the fluorochrome, Hoechst 33258. The fluorescence intensity of each chromosome was measured in a flow cytometer with a light source tuned to excite the Hoechst dye. Each peak in the distribution represents the measurements of an individual chromosome type. As in figure 1.7, the position of the peak contains information on the DNA content of each chromosome type, and the area under each peak reflects the relative frequency of each chromosome type in the original cells.

and high resolution flow cytometers, it is possible to resolve 21 of the 24 different human chromosome types in a flow karyotype (chapter 7).

Several properties of flow cytometry make it a unique tool for cell biologists. The optical properties of individual cells or chromosomes are analyzed. These properties are expressed in a quantitative and objective manner. High measurement precision allows the detection of small differences in the number of fluorescent molecules bound to each particle in a suspension. Furthermore, the measurements can be performed rapidly. Analysis rates of a 1000 events per second are normal, and rates up to 20,000 events per second have been reported. In addition, particles can be sorted from the others in a suspension on the basis of their optical properties for subsequent biochemical or biological assays. Chromosomes, for example, can be purified to be used in gene mapping or cloning experiments.

The application of flow cytometry to the study of cells and chromosomes is constrained by several conditions. To be quantified, a given biological property must be translated into an optical phenomenon, such as fluorescence or scattered light. This point implies that a component of interest can be quantified flow cytometrically only when it can be suitably converted into an optical phenomenon. Fluorescent labels

and assays have allowed the flow cytometric quantification of such diverse cellular properties as intracellular pH, membrane fluidity, DNA content, protein content, and the presence of membrane antigens. The second constraint is that the cells or chromosomes must exist as individual entities in suspension. This places restrictions on the handling of the particles prior to analysis. Many labeling procedures have been developed for cells or chromosomes fixed firmly to slides. Without this structural support, the particles would fall apart during many procedures. These labeling procedures must be adapted to suspension. Thirdly, to glean relevant biological information from the fluorescence signals of each particle, these signals must be accurately quantified. Variation in the fluorescence intensities of identical particles must be minimized through optimal preparation and and staining.

This thesis deals with the application of flow cytometry to the study of the structure and molecular organization of nuclei and chromosomes. This thesis presents successful attempts to expand upon the properties of chromatin that can be fluorescently labeled for quantification using flow cytometry. Specific structural proteins complexed with DNA, the histones, are quantified in the chromosomes of several mammalian species. A technique is presented for the stabilization of chromatin in nuclei and chromosomes. This allows in situ hybridization to be performed in suspension. An exciting consequence of this is the detection of specific DNA sequences in interphase nuclei using dual beam cytometry. In addition, detailed studies of human chromosomes are presented. The properties that are analyzed include DNA content, base composition and interaction between DNA-specific dyes. The results presented here also show that chromosomes derived from clinical material can be analyzed on the basis of these properties. The first flow karyotypes of fetal cells from human amniotic cell cultures are shown.

This thesis is organized as follows.

In the first section, the basic principles of flow cytometry are described. The application of flow cytometry to the analysis of chromosomes and their content is summarized. The factors important for flow cytometric resolution and staining are delineated.

In section 2, flow cytometry is used to examine the gross differences among the chromosomes from a range of human cell types, as well as from Chinese hamster cells. Total DNA content and average base composition are the properties quantified to discriminate the chromosomes. Single and dual laser flow cytometers are employed to produce uni- and bivariate

fluorescence distributions. This series of papers includes the results of efforts to increase resolution through improvements in the isolation and staining of chromosomes. Important is a new chromosome isolation procedure, which yields stable chromosomes and high flow karyotype resolution. The base composition of chromosomes is analyzed as part of a study of the binding kinetics and interactions of a variety of DNA stains.

In the third section, the structural organization of individual chromosomes is analyzed. The size of DNA fragments obtained from isolated chromosomes is presented. Structures that can be stained to produce conventional banding patterns are shown to be maintained in isolated chromosomes. One structural protein, histone 2B, is quantified in single chromosomes using immunofluorescent labeling and flow cytometry. This study also provides some insight into the structural conformation of individual chromosomes.

In the fourth section, chromosomes and nuclei are examined at the level of the nucleotide base sequence in the DNA. Techniques are described in these chapters for the stabilization of chromosomes and nuclei in suspension. With this stabilization, chromosomes and nuclei can be subjected to conditions suitable for DNA denaturation and for high stringency DNA-DNA hybridization. The binding of an antibody specific for bromodeoxyuridine in single-stranded DNA demonstrates the degree of strand separation in chromosomes and is quantified flow cytometrically. In situ hybridization techniques are applied to stabilized mouse interphase cell nuclei in suspension. After an immunofluorescent labeling procedure, the relative amount of mouse satellite DNA sequences in these nuclei is quantified in a dual beam flow cytometer. An alternative means for the detection of specific nucleic acid sequences in isolated nuclei and chromosomes is also presented in this section. Chromosomes and nuclei are bound to nitrocellulose filters where hybridization can be performed and can be visualized with an immuno-enzymatic procedure. Of special interest is the observation that the hybridization of DNA sequence probes can be visualized for single nuclei on these filters.

At each of these levels of genomic organization, flow cytometry is an ideal tool for quantifying the properties of chromosomes and nuclei. The potentials for applying flow karyotyping to the study and diagnosis of genetic abnormalities in human hereditary disorders and cancers are increased with the findings presented in this thesis. In this regard, the analysis of chromosomes from fetal cells obtained from amniotic cell cultures and of chromosomes from peripheral blood lymphocyte cultures are

promising. The enrichment of mitotic cells from the bone marrow of normal rats presented here may also advance the application of flow karyotyping to the chromosomes of this cell type. The techniques described for in situ hybridization in suspension in combination with dual beam flow cytometry open many avenues of research. The detection and quantification of numerical chromosome abnormalities, gene amplification or viral sequences in individual human cells may be possible. The hybridization techniques in suspension and on filters may have consequences for determining the frequency of cells containing specific DNA sequences in a heterogeneous population. In the final chapter, the prospects for these and other areas of future research are discussed in light of present limitations.

SECTION I

BACKGROUND INFORMATION: CHROMOSOME ANALYSIS WITH FLOW CYTOMETRY AND FLUORESCENT STAINS

Introduction

The flow cytometric approach is placed in perspective with other techniques available for the study of chromosome organization in chapter 2. In this chapter, the properties of chromosomes that can be determined with this tool are described. The use of flow cytometry in karyotyping and in gene mapping studies is outlined. This chapter also describes how chromosome fractions, purified by flow sorting, have been utilized in studies of the genomic organization.

In chapter 3, a survey is made of the procedures available for the isolation of chromosomes for flow karyotype analysis. The requirements for a good chromosome isolation procedure are outlined. The differences, limitations, and specific advantages of the available procedures are compared.

The flow cytometers themselves are the subject of chapter 4. The features of three flow cytometers serve as the basis for this discussion. These three are a single laser machine, a dual laser machine and, finally, a 3-laser machine in the development stage. Aspects of flow cytometric analysis, including illumination, sample flow, fluorescence collection, signal processing and data acquisition, are addressed. The technical developments that have improved measuring accuracy and sensitivity or may have the potential to do so, are described in this chapter.

The characteristics of the fluorescent dyes used in chromosome analysis are discussed in chapter 5. The features of the ideal dye for flow cytometry are outlined. The structures, binding characteristics, and absorption and fluorescence spectra of a series of fluorescent biological dyes are presented.

Chapter 2

CHROMOSOME ANALYSIS AND SORTING WITH FLOW CYTOMETRY

The first flow cytometric measurements of the DNA content of individual chromosomes were presented in 1975 (Gray et al., 1975a, b; Stubblefield et al., 1975), approximately six years after the introduction of flow cytometry for the analysis of cells and whole nuclei (Van Dilla et al., 1969). Because the usefulness of flow cytometry in karyotype analysis and in the purification of chromosomes depends on the degree to which chromosomes are resolved, much effort has been placed on improving the quality of the measurement. Since 1975, many advances have been made in the isolation procedures (see chapter 3), the fluorescent DNA-specific dyes (chapter 5), and the flow cytometers themselves (chapter 4). Chromosomes from an increasing number of species and cell types can now be analyzed in a flow cytometer. Using commercially available machines equipped with one laser, virtually all of the chromosomes of the Chinese hamster (figure 1.8) and approximately 17 different chromosome groups from human cells can be discriminated (figure 2.1). More elaborate machines, with two lasers tuned to different wavelengths, allow the use of two fluorescent stains that bind with different base pair specificities to the DNA in chromosomes. The binding of these stains can be analyzed separately. With this approach, at least 21 of the 24 human chromosome types can be distinguished (figure 2.2 and chapter 7). Flow cytometers with sorting capabilities can be used to isolate individual chromosome types for microscopical or biochemical analyses.

These advances have made it possible to introduce flow karyotype analysis in a range of research areas (reviewed by Lebo (1982) and Young (1984)). The flow karyotype can be analyzed to detect chromosomal abnormalities of possible importance in medical genetics, in tumor biology, or in the study of the effects of radiation and chemical mutagens. Flow cytometry can be used to simplify and speed the assignment of genes to their positions in chromosomes. Flow cytometry can also be used to purify individual chromosome types for further biochemical analysis or for the construction, using recombinant DNA techniques, of chromosome-enriched genomic libraries. This chapter is intended to give a summary of these applications.

Karyotyping and the detection of chromosomal abnormalities

Traditionally, karyotyping and the detection of chromosomal rearrangements are performed by banding analysis. For banding studies, metaphase cells are collected and fixed to microscope slides. The chromosomes in them are then stained to produce intricate banding patterns (See Pearson, 1973; Pearson and van Egmond-Cowan, 1976; Latt, 1976; Yunis, 1983). These bands can be used to identify the chromosomes. Changes in chromosome size, number, or banding pattern reflect chromosomal defects such as deletion, translocation, inversion or aneuploidy.

In the clinic, this technique has been successfully applied to the early diagnosis of congenital disorders in amniotic cells, such as trisomy of chromosome 21 and sex-linked hereditary defects (Golbus et al., 1979; Fuchs, 1980; Ledbetter, 1983). The presence of characteristic chromosome rearrangements have also been used in the diagnosis and prognosis of various neoplasias, particularly those of the haemopoietic system (reviewed by Sandberg and Hossfeld, 1970; Rowley, 1973; Yunis, 1983; Zech et al., 1976; LeBeau and Rowley, 1984; Van den Berghe, 1984).

The examination of banded chromosomes under the microscope is time consuming, and it requires the experience of a trained cytogeneticist. The technique is limited to the detection of aberrations involving at least one band width of chromatin. Variability in chromosome staining and morphology

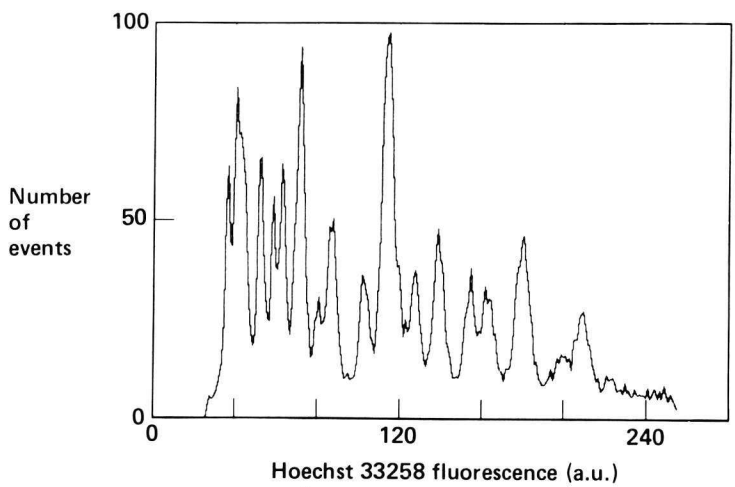


Figure 2.1. Frequency distribution of the fluorescence intensity of human chromosomes stained with Hoechst 33258 and measured in a single laser flow cytometer. The chromosomes were isolated from the mitotic cells collected from phytohemagglutinin-stimulated peripheral blood lymphocyte cultures using the procedures described in chapter 7.

from cell-to-cell on the slides can result in subjective diagnoses, which limit interlaboratory comparisons. Aberrations that occur with low frequencies require inspection and karyotyping of a large number of cells.

Analysis of the specific electrophoretic pattern of the restriction enzyme fragments of cellular DNA (Southern, 1975) is also used to detect genetic rearrangements or polymorphisms in a cell population (Kan and Dozy, 1978; Little et al., 1980; de Klein et al., 1982; Taub et al., 1982). Small changes in the DNA sequence, involving less than one band width, can be detected with this technique. Because the total amount of DNA isolated from a population of cells is used as starting material, this technique does not allow the detection of infrequently occurring defects or the localization of defects to specific chromosomes.

The limitations of these techniques have led to efforts to automate the analysis of metaphase cell preparations for genetic defects. Scanning cytophotometers have been developed that are capable of measuring the DNA content and centromeric index of chromosomes from individual cells with high precision (Mendelsohn et al., 1973, Van der Ploeg et al., 1977a, b; Geraedts and Van der Ploeg, 1980; Mayall et al., 1984). However, processing the visual image is a relatively slow process and is hampered by staining variability among different cells and slides. Although it has been used to quantify karyotype variation among individuals and the amount of DNA involved in translocations (Mayall et al., 1977; Carrano et al., 1979c; Geraedts and Van der Ploeg, 1980), this technique must be, as yet, always performed in conjunction with conventional banding analysis (Mayall et al., 1984).

Chromosome analysis with flow cytometry

Chromosomes can be classified and chromosome abnormalities can be detected using flow cytometry. The main advantages of flow cytometry include the ability to measure fluorescent properties of individual stained chromosomes quantitatively and objectively. The high rate of measurement (1000's per second) allows the chromosomes from a large number of cells to be analyzed in a short time. This increases the likelihood that defects occurring at low frequencies in a cell population can be detected. Variation among cells or chromosomes in staining intensity is reduced by the ability to analyze particles suspended in equilibrium with dyes. The analysis of large numbers of particles contributes to the statistical accuracy of the measurement. Furthermore, stain combinations can be chosen such that the fluorescence measurements yield information on the DNA content and the chemical composition of chromosomes.

Chromosomes are isolated from a suspension of mitotic cells by swelling the cells in a hypotonic solution and disrupting the plasma

membrane to release the chromosomes. The chromosomes must be released into a solution containing agents that stabilize the chromatin in suspension. The chromosomes, stained with one or more DNA-specific fluorescent dyes, pass one-by-one through the illumination beam of a laser in the flow cytometer (van Dilla et al., 1969). The fluorescence signals emitted by each chromosome are detected, amplified and collected in a multi-channel histogram. Since thousands of chromosomes pass through the illumination beam each second, a fluorescence distribution representative of the chromosome population can be constructed in a few minutes.

Fluorescence distributions

The fluorescence distribution of a suspension of isolated human chromosomes is shown in figure 2.1. The flow measurements contain quantitative information on the relative DNA content and the relative frequency of the chromosomes. Each peak in the distribution represents a distinct group of chromosomes. The peak mean is approximately proportional to the amount of stain bound. The area under each peak is a measure of the frequency of occurrence of the chromosome type in the total suspension. The peak width is a measure of the uncertainty of the measurement. A background continuum produced by chromosome clumps, broken chromosomes and debris underlies the peaks.

Changes in the size or in the base composition of chromosomes (resulting, for example, from a deletion or translocation) that occur in the entire cell population should be reflected, in theory, in peak position shifts and peak area changes in the fluorescence distribution. A numerical chromosome abnormality present in all the cells being analyzed (for example, trisomy) is predicted to result in a change in the area of the peak of the chromosome concerned. An increase in the level of the background continuum is expected from random breaks or other heterogeneously occurring chromosome aberrations. Unique changes in chromosomes, occurring in only a few cells in the population (mosaicism), could be detectable in principle, if the newly formed peaks are well-separated from the others in the distribution.

Flow karyotyping

The position of the chromosomes in the fluorescence distribution concurs with conventional cytogenetic size discrimination. Fluorescence distributions can, therefore, be used as karyotypes. This has been determined by sorting the chromosomes responsible for each peak and identifying them by conventional banding techniques. The peaks in the flow karyotypes of the Chinese hamster (Gray et al., 1975a, b; 1979a; Carrano et

al., 1979b; 1978) mouse (Disteche et al., 1981) and Indian muntjac (Carrano et al., 1976) have been identified in this way.

High resolution single laser measurements have also been made of human metaphase chromosomes. In this way, up to 16-17 different chromosomes groups can be distinguished, which also correlate with conventional cytogenetic classification (Gray et al., 1979b; Carrano et al., 1979a, b; Yu et al., 1981; Sillar and Young, 1981; Young et al., 1981; Matsson and Rydberg, 1981; Lebo and Bastian, 1982; Krumlauf et al., 1982; Fantes et al., 1983a; Lalande et al., 1984; Young, 1984; and chapter 7).

Unfortunately, not all of the human chromosomes are discriminated in a univariate flow karyotype. However, when two fluorescence properties are measured for each chromosome in a dual laser machine (Dean and Pinkel, 1978), 20-21 chromosome groups can be discriminated in the resulting bivariate fluorescence distribution (figure 2.2) (Gray et al., 1979b; Carrano et al., 1979b; Langlois et al., 1982; Lebo and Bastian, 1982; Lebo et al., 1984; Collard et al., 1984; Meyne et al., 1984; Lalande et al., 1985; and chapter 7-9). This approach makes use of two dyes, which differ in their affinity for certain base pairs in DNA and which exhibit different fluorescence excitation and emission spectra. A common dye combination is Hoechst 33258 (HO, with a high affinity for adenine-thymidine base pairs (Latt and Wohlleb, 1975)) and chromomycin A3 (CA3, specific for guanine-cytosine base pairs (Ward et al., 1965)). Differences in the Hoechst to chromomycin fluorescence ratio have been correlated to differences in base composition (Langlois et al., 1980). With this approach, only chromosomes 9 through 12 are unresolved. The AT-specific DNA stains, DAPI or DIPI (Lin et al., 1977), can be used to further discriminate chromosome 9 from chromosomes 10-12 (Lebo et al., 1984; Meyne et al., 1984). Slit scan flow cytometry, in which a fluorescence intensity profile is measured along each chromosome (Cram et al., 1979; Gray et al., 1979c, 1980), also has been shown to resolve the chromosomes in this group (Lucas et al., 1983).

Homologue differences in certain chromosomes of the Chinese hamster (Gray et al., 1979b; Carrano et al., 1979b, Langlois et al., 1980; chapter 6, 7) and of man (Gray et al., 1979b; Carrano et al., 1979b; Lebo and Bastian, 1982; chapters 7, 9) can also be detected in a bivariate fluorescence distribution.

Peak variation

It is necessary to assess normal variation in peak position and area and the degree of normally occurring polymorphisms before automated classification of flow karyotypes and the detection of abnormalities is possible (see chapters 7 and 8). The isolation procedure must not, in

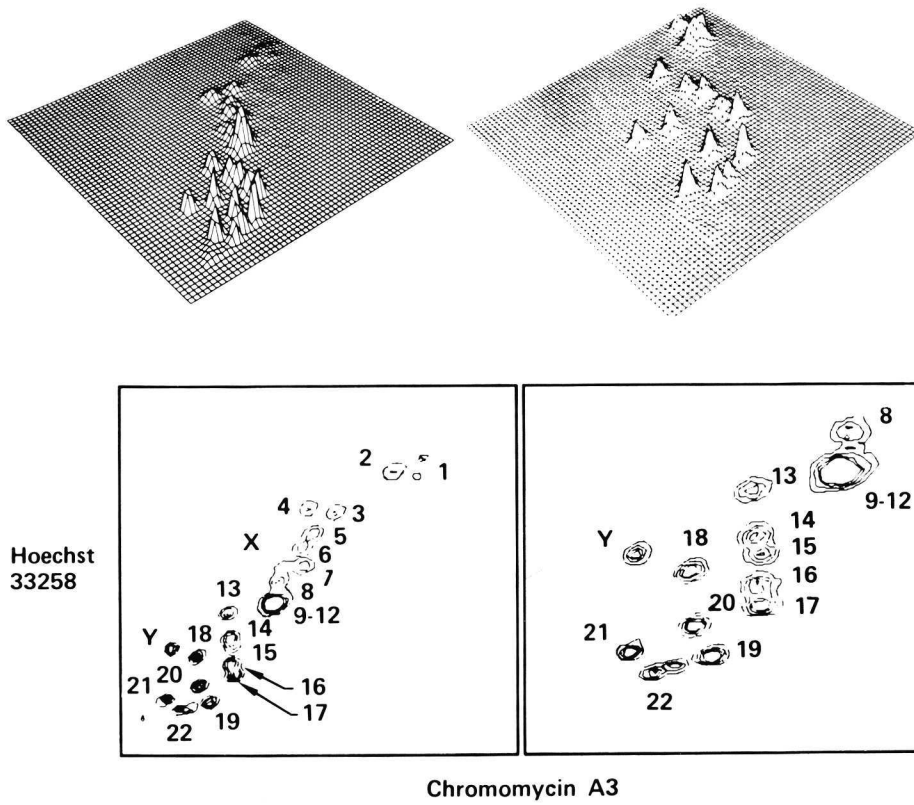


Figure 2.2. Bivariate frequency distribution of human chromosomes stained with Hoechst 33258 and chromomycin A3 and measured in a dual laser flow cytometer. Hoechst fluorescence is plotted along the ordinate; chromomycin fluorescence is plotted along the abscissa. In the isometric plots at the top, the frequency of chromosomes with a given Hoechst and chromomycin A3 fluorescence is indicated by the height of the peak along the Z-axis. In the bottom panels, the frequency is indicated by contour lines drawn at equal heights on the frequency axis. In the left panels in both representations, all human chromosomes are displayed. In the right panels, only the smaller chromosomes (8-22, Y) are displayed by increasing the amplification during measurement. Note that all chromosomes, except those in the 9-12 group, are well resolved. The chromosomes were isolated from normal human fibroblasts cultures using the procedures described in chapter 7.

itself, produce aberrations such as clumping and debris or result in under-representation of certain chromosome groups. In an early study, Carrano and coworkers (1979b) showed that variation in peak means as low as 1-2.5% could be obtained for Chinese hamster chromosomes prepared over an extended period of time. Studying a series of human peripheral blood samples, Young et al. (1981) detected slight variability, attributable to heterochromatin differences, in the peak means of several chromosomes. This was especially the case with the Y chromosome. Fantes et al. (1983a) capitalized on this normal variability to select an individual with a Y chromosome well separated from the other chromosomes in a univariate distribution to sort out the Y chromosome for the construction of a Y-specific recombinant library. With dual beam cytometry, Langlois et al. (1982) studied the variation in peak position in a bivariate flow karyotype of chromosomes isolated from the lymphocytes of ten normal individuals. They determined that the sample-to-sample and run-to-run variation in the flow karyotypes of each individual was much less than that among different individuals. The variation among individuals was sufficiently low that 95% confidence ellipses containing the peak means of each chromosome of 10 individuals did not overlap (except for chromosomes 9-12 and 14, 15). Identification of the chromosomes responsible for the peaks in the flow karyotypes was possible on the basis of peak position alone. These authors also determined that the low variability and the high precision in peak mean location (0.5% variability) would allow the detection of homogeneously occurring rearrangements as small as 1/600th of the mitotic genome (one band width).

Detection of chromosome abnormalities

With this foundation of high precision and low variability, flow karyotype analysis has been applied to the detection of chromosomal abnormalities. Chromosome aberrations can be classified into three categories: 1) homogeneously occurring aberrations in all cells in a population, 2) heterogeneously or randomly occurring aberrations, 3) mosaicism: unique aberrations occurring in a few cells of the population.

Homogeneously occurring chromosome aberrations have been associated with human cancers with conventional techniques. With flow cytometry, characteristic chromosomal abnormalities have been detected in the flow karyotypes of a Chinese hamster cell line (Gray et al., 1975a; Carrano et al., 1978), a human colon carcinoma cell line (Gray et al., 1984; chapter 7), a rat rhabdomyosarcoma cell line (Kooi et al., 1984), several Burkitt's lymphoma cell lines and a chronic myeloid leukemia line (Wirchubsky et al., 1983). Lalande et al (1985) reported the detection of an inverted duplicate of chromosome 15 using Hoechst, chromomycin and netropsin

staining. Flow cytometry has been used to follow karyotype changes in Chinese hamster cell cultures associated with spontaneous transformation (Cram et al., 1983). Chromosomal aberrations maintained in culture after exposure of Chinese hamster cells to 10 Gy X-rays have been detected in a flow karyotype (Carrano et al., 1978).

The ability to isolate chromosomes from amniotic cells (chapter 9) has made it possible to correlate the flow karyotypes of these cells with conventional cytogenetic analysis (Gray et al., personal communication). Trisomy of chromosome 21 (Down's syndrome) has been detected flow cytometrically (Gray et al., personal communication).

The quantification of abnormal chromosomes such as dicentrics and acentric fragments, which occur in some cells of individuals exposed to radiation or to chemical clastogens, is important in assessing exposure to these environmental hazards (Lloyd, 1983). The speed with which the chromosomes from a large number of cells can be analyzed in a flow cytometer makes this technique interesting for screening for these aberrations in populations at risk. The ability to prepare high quality flow karyotypes from peripheral lymphocyte cultures (Yu et al., 1981; Young et al., 1981, Matsson and Rydberg, 1981, chapter 7) should also aid in population screening studies.

Damage to Chinese hamster chromosomes resulting from exposure to X-rays (Carrano et al., 1978; 1979b; Gray et al., 1975b, Aten et al., 1980; Fantes et al., 1983b) or UV irradiation in combination with caffeine exposure (Cremer et al., 1982a) has been correlated to the height of the debris continuum underlying the chromosome peaks in a fluorescence distribution. Doses of X-rays as low as 0.5 Gy are reflected in an increased debris continuum (Aten et al., 1980). High numbers of dicentrics and chromosome fragments found in sorted fractions indicate that these abnormal chromosomes contribute to this continuum (Van Dilla et al., 1976; Carrano et al., 1978). Changes in the coefficient of variation of the peak representing the largest Chinese hamster chromosome has also been used as a measure of chromosome damage (Otto and Oldiges, 1980). Slit scan flow cytometry has been used to determine the frequency of dicentric chromosomes occurring in Chinese hamster chromosomes exposed to X-rays (Gray et al., 1984).

To determine the level of mosaicism detectable with flow cytometry, Carrano and coworkers (1979b) mixed two clones of Chinese hamster cells. They determined that, if the aberrant chromosomes of the X-irradiated clone (3 Gy) were well resolved in the karyotype, it was possible to detect them when present at a frequency as low as 5%.

Chromosome purification using flow cytometry

Flow cytometry can also be used as a preparative tool to collect fractions of individual chromosome types. These chromosome suspensions can be analyzed biochemically for nucleic acid and protein content or for the presence of specific gene sequences. They can also be used as the starting material for cloning experiments. Other fractionation techniques, such as velocity sedimentation (Collard et al., 1981) or density gradient equilibrium centrifugation (see review by Hanson, 1975) can be used to obtain suspensions enriched in specific chromosomes. However, these techniques are overshadowed by the resolving power of the fluorescence measurements and the purity of the chromosome fractions obtained with flow cytometry. Chromosome suspensions as pure as 80-99% have been reported using flow sorting (Carrano et al., 1976; 1979a, b; Sawin et al., 1979; Lebo and Bastian, 1982; Fantes et al., 1983a; Cremer et al., 1984) Furthermore, the basis for fractionation can be extended with flow cytometry from chromosome size to other chromosomal properties, such as base composition, protein content, or eventually to the presence of specific DNA sequences (chapter 14).

The advantage of bulk separation techniques, however, is the ability to fractionate large numbers of chromosomes in a single step. Some laboratories use bulk fractionation as a pre-enrichment step before sorting with flow systems (Stubblefield and Wray, 1978; Stubblefield and Oro, 1982; Collard et al., 1980a; 1980b; 1984). The efficiency of flow sorting has been improved with the recent development of a flow cytometer capable of sorting more than 20,000 chromosomes per second (Gray et al., 1981; Peters et al., 1985).

Gene mapping with flow cytometry

The chromosomal locations of genes can be determined by a variety of methods. Gene location can be assigned by family linkage analysis (McKusick, 1980) or by studying segregation in somatic cell hybrids (Ruddle and Creagan, 1975). Another approach is in situ hybridization, the hybridization of labeled probes to chromosomes on slides (Gall and Pardue, 1969; John et al., 1969). With this technique, single copy genes have been localized to segments of chromosomes (Gerhard et al., 1981; Harper and Saunders, 1981; Landegent et al., manuscript submitted).

Genes can also be localized by hybridizing specific DNA sequence probes to fractions of chromosomes enriched for certain chromosome types by flow sorting. In one approach, the DNA of sorted chromosomes is extracted and subjected to restriction enzyme digestion. The hybridization of ^{32}P-labeled cDNA probes to the DNA fragments, separated in a Southern blot, reveals the chromosomes containing the genes being assayed

(Carrano et al., 1981; Krumlauf et al., 1982). Sublocalization of genes to the short arm of chromosome 11 has been shown using sorted chromosomes from a series of cell lines containing different translocations of this chromosome (Lebo et al., 1979; 1982).

In another approach, chromosomes are sorted directly onto nitrocellulose filters where they are denatured, bound, and hybridized to gene probes of interest. Chromosome-specific probes can be used in this technique to determine the chromosomes responsible for the peaks in the flow karyotype (Bernheim et al., 1983; Collard et al., 1985; see chapter 15). This eliminates the need to sort and band chromosomes. Lebo and coworkers (1984) used a dual laser flow sorter to sort 21 different human chromosome groups onto separate spots on a filter. Genes for which probes are available can be mapped to any of the 21 chromosomes groups in a single hybridization step. Gene probes can also be hybridized to translocated chromosomes sorted onto filters to determine the location of genes in these chromosomes. The chromosomal location of several oncogenes have been determined in this way (de Taisne et al., 1984; Lebo et al., 1984; Collard et al., 1985).

Flow cytometry can also be used to quantify specific DNA sequences of interest in nuclei after in situ hybridization in suspension (chapter 14). The results in chapter 13 indicate that it may be soon possible to quantify the hybridization signal in individual chromosomes.

Chromosome-enriched genomic libraries

Genomic libraries are useful as a source of probes to study genomic organization, genetic polymorphism and gene regulation in normal cells. The probes are also useful in the study of the nature of genetic abnormalities associated with human disorders. The genomic changes that have occurred during evolution can also be studied.

Flow cytometry and sorting have been used successfully for the construction of the genomic library of various chromosomes. Cell lines are often selected specifically to allow discrimination of the desired chromosome in a flow karyotype. Genomic libraries have been constructed for the mouse X chromosome (Kunkel et al., 1982) and the Cattanach translocated form of the mouse X chromosome (Disteche et al., 1982). The following human chromosomes have been sorted to produce chromosome specific libraries: chromosome 6 (Boncinelli et al., 1984); 21 and 22 (Krumlauf et al., 1982); X (Davies et al., 1981), various portions of the X chromosome, using cell lines containing a dicentric X and an isochromosome of the long arm of X (Lalande et al., 1984); the Y, using a univariate Hoechst fluorescence distribution (Fantes et al., 1983) or using dual beam flow cytometry and a Chinese hamster/ human hybrid retaining the human Y

chromosome (Cremer et al., 1982b; 1984; Muller et al., 1983); and chromosome 1, from neuroblastoma cells containing regions of amplified genes (Kanda et al., 1983). The probes produced from the latter library were used to study transposition and function of this amplified sequence in neuroblastoma lines.

Two laboratories in the USA have joined forces to prepare recombinant libraries that contain sequences unique to each of the human chromosomes. The Livermore and Los Alamos National Laboratories are both equipped with flow cytometers capable of discriminating over 20 groups of chromosomes and sorting at rates up to 20,000/s (Peters et al., 1985). Chromosome suspensions are prepared from human/Chinese hamster hybrids and human fibroblast lines using the isolation procedure described in this thesis (chapters 6,7). Approximately $4x10^6$ copies of each chromosome are sorted and cloned. To date, this project has resulted in recombinant DNA libraries for 13 of the human chromosomes (Deaven and Van Dilla, 1985).

Chapter 3

CHROMOSOME ISOLATION PROCEDURES

Introduction

A high degree of chromosome resolution is required for the optimal application of flow cytometry to karyotype studies, chromosome aberration detection, chromosome purification, and gene localization. Ideally, each human chromosome type is resolved into a distinct peak in the flow karyotype, and small changes in chromosome composition are detectable. The resolution of chromosomes in a flow karyotype depends on the chromosome isolation procedure, the fluorescent staining, and the precision of the measuring instrument. In the next chapter, the flow cytometers used in chromosome analysis and their modifications are discussed. The characteristics of the fluorescent dyes used in chromosome analysis are presented in chapter 5. In this chapter, the requirements for optimal chromosome isolation are described and the various chromosome isolation procedures in current use are compared.

Requirements

The ideal chromosome isolation procedure produces a suspension of single chromosomes, without clumps or debris. The chromosomes should be stable in suspension. Little chromosome damage or composition change should occur. Chromosomes should not be selectively lost from the suspension. The method should be reproducible, and variability in the relative chromosome peak positions and areas should be low in repeated measurements for the same individual. It should be possible to fluorescently label the chromosomes with a variety of DNA and protein stains. The fluorescent staining should be homogeneous, stable, and should also show low variability. In addition, the method should be simple. For clinical studies, the isolation procedure should perform well with mitotic cells from a variety of cell types. Chromosome yield should be high. Storage of chromosomes before flow cytometric analysis should not result in a degradation of the fluorescence distributions.

In addition, certain applications place specific demands on the chromosome isolation procedure used. For example, a low debris level and the ability to isolate chromosomes from an easily accessible cell type, such as peripheral blood lymphocytes, are necessary to correlate chromosome

damage to the exposure of individuals to mutagenic agents. For the construction of genomic libraries and for gene mapping studies, it should be possible to isolate DNA fragments of a large size from sorted chromosomes. For studies of chromosome structure, the biochemical composition and the morphology of isolated chromosomes should closely resemble that of chromosomes in situ. For many applications, the ability

Table 3.1

THE BASIC STEPS IN CHROMOSOME ISOLATION FOR FLOW CYTOMETRY

1. Mitotic arrest
2. Mitotic cell collection
3. Swelling in hypotonic solution
4. Solubilization of plasma membrane with detergent
5. Physical disruption of cells to release chromosomes
6. Stabilization of chromosomes in suspension
7. Staining with fluorescent dyes

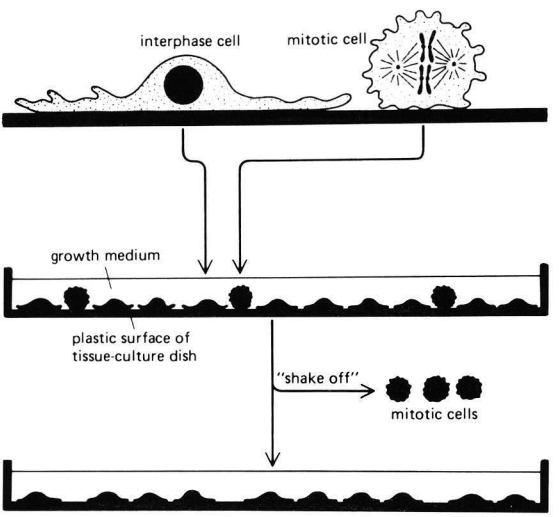


Figure 3.1. Mitotic cell collection from fibroblast monolayer cultures. At mitosis, the cells round up and loosen from the bottom surface of the culture flasks. The mitotic cells can be collected in the growth medium by shaking the flask (reprinted with permission from Alberts et al., 1983).

to identify sorted chromosomes by conventional banding techniques is desirable.

General features of the isolation procedures

Several chromosome isolation procedures have been developed specifically for flow cytometry in the past ten years. Using two DNA fluorochromes and dual laser flow cytometry, it is possible to resolve 20 to 22 chromosome groups in chromosome suspensions prepared from human mitotic cells with a variety of procedures: the hexylene glycol method (Langlois et al., 1982), the citric acid method (Collard et al., 1984), the polyamine method (Lebo et al., 1984; Lalande et al., 1985), and the Hepes/$MgSO_4$ method (chapter 7); Meyne et al., 1984).

The chromosome isolation procedures have common features as outlined in table 3.1. The details of the procedures are presented in table 3.2. Cells are arrested in mitosis by treating cell cultures with an agent, such as colchicine, that blocks the formation of the mitotic spindle apparatus. The mitotic cells are collected by shaking them from fibroblast culture layers (figure 3.1) and concentrating them by centrifugation, or by spinning down entire suspension cultures. The cells are swollen in a hypotonic solution. This spreads the chromosomes within the cells, so that they can be better dispersed when the cell is disrupted. Detergents and/or physical disruption are used to break open the swollen cells and release the chromosomes into a suspension. A stabilizing agent must be present in the solutions to maintain compact and intact chromosomes. Before flow cytometric analysis, the chromosomes are stained with fluorescent dyes.

The procedures in detail

Within this framework of basic steps, the chromosome isolation procedures vary in the mode of chromosome stabilization, the number of mitotic cells needed for optimal results, their applicability to different cell types, their suitability for the production of banding patterns in chromosomes sorted and fixed to slides, and in the size of the DNA fragments that can be extracted from the isolated chromosomes. The procedures are compared in the discussion of chromosome preparation presented in the following pages.

Chromosome stabilization

Crucial to any chromosome isolation procedure is the stabilization of chromosomes in suspension. Figure 3.2 shows an example of what can be expected if stabilizing agents are omitted from the chromosome isolation buffers. Early chromosome isolation procedures utilized low pH and divalent cations to stabilize chromosome structure for morphological and

biochemical studies (reviewed by Hanson, 1975). Low pH isolation methods have been used for flow cytometric analysis of chromosomes in several laboratories (Stubblefield and Wray, 1978; Stubblefield et al., 1975, Collard et al., 1980a; Stöhr et al., 1982 (in combination with the hexylene glycol method)). A problem with these methods is the lability of chromosomal proteins and damage to nucleosomal structure in isolated chromosomes at low pH (Darzynkiewicz et al., 1984; Tien Kuo, 1982). Therefore, efforts have been made isolate chromosomes in neutral pH (Maio and Schildkraut, 1967).

Various agents have been used to prevent the swelling and degradation of chromosomes in suspension at neutral pH and have been used with success in flow cytometry. The stabilizing agents include:

1. Divalent cations, such as magnesium and calcium ions. These ions have been shown to increase the melting temperature of DNA (Eichhorn, 1962; Baba and Kagemoto, 1974; Darzynkiewicz et al., 1975; 1976), especially at low ionic strengths (Tabor, 1962; Zimmer et al., 1971). These ions also keep chromatin condensed (Garrett, 1971; Olins and Olins, 1972; Aaronson and Woo, 1981; Zelenin et al., 1982; Marsden and Laemmli, 1979; Hearst and Botchan, 1970; Cantor and Hearst, 1970; Maio and Schildkraut, 1967). Several chromosome isolation buffers used for flow

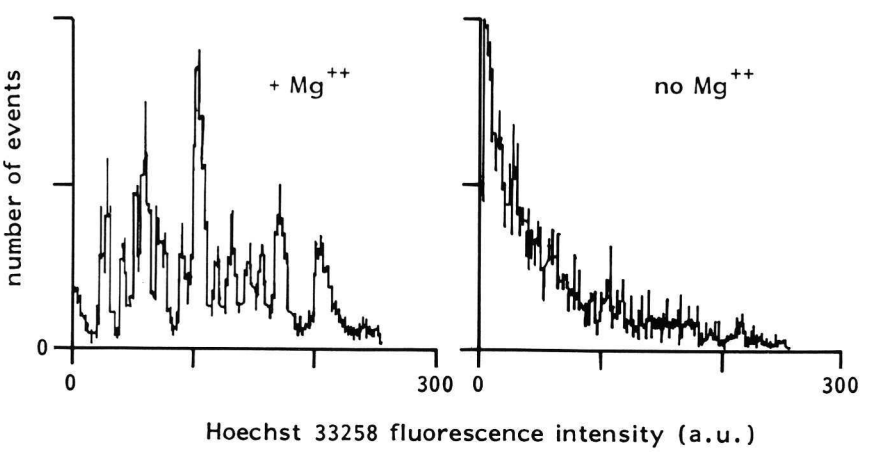


Figure 3.2. Fluorescence distribution of human chromosomes measured in the presence and absence of the chromosome-stabilizing agent, Mg^{++}. The chromosomes were prepared from normal human fibroblasts as described in chapter 7 with (A) or without (B) the addition of 10 mM $MgSO_4$. The chromosomes were then stained with Hoechst 33258 and measured in a flow cytometer.

cytometry incorporate one or both of these ions (Otto et al., 1980; Bijman, 1983; chapter 6).

2. Cationic polyamines, such as spermine and spermidine. These compounds raise the thermal stability of DNA and prevent mechanical damage to isolated DNA (Tabor, 1962). A chromosome isolation procedure incorporating these agents was developed by Blumenthal et al. (1979) and modified for flow cytometry by Sillar and Young (1981) and Lalande et al. (1984).

Divalent cations and polyamines presumably stabilize the DNA helix by binding to the negatively-charged phosphate groups on the outer periphery of the DNA helix and thereby increasing the effective strength of the Van der Waal's and hydrogen-bonding forces that hold the two strands together (Tabor, 1962). The affinity constant for binding of spermidine to DNA is higher than that of Mg^{++} ions (Tabor, 1962).

3. Hexylene glycol. A chromosome isolation procedure using this agent was developed by Wray and Stubblefield (1970) and has been used for flow karyotyping with the addition of divalent cations (Carrano et al., 1979b; Gray et al., 1979b). An isolation solution containing hexylene glycol and calcium ions and buffered at pH 10 has been reported by Wray et al. (1972).

4. Propidium iodide (PI). Intercalating DNA stains such as PI are known to stabilize the DNA helix (Le Pecq and Paoletti, 1967). Isolation procedures employing PI as the stabilizing agent have been used for flow karyotyping (Aten et al., 1980; Cram et al., 1983; Matsson and Rydberg, 1981).

5. 4,5',8-trimethylpsoralen. This nonfluorescent intercalating agent has been used to stabilize chromosomes for flow cytometric measurement by Yu et al. (1981).

Mitotic cell collection

The length of the mitotic cell block depends on the growth characteristics of the cell line being used. Routinely, cells with 20 h doubling times (such as human fibroblasts) are incubated in the presence of a spindle poison for 14-16 h; cell lines with shorter doubling times (e.g., Chinese hamster fibroblasts: 13 h) are blocked only 4-6 h.

Early cell culture passages have higher mitotic indices and give higher mitotic cell yields than later passages (Lebo, 1982). Karyotypic

Table 3.2. CHROMOSOME ISOLATION PROCEDURES

HEXYLENE GLYCOL (Wray and Stubblefield,1975; modified by Yu et al., 1981)	PROPIDIUM IODIDE (Aten et al., 1980; Buys et al., 1982)	POLYAMINE (Blumenthal et al., 1979; Sillar and Young, 1981; Lalande et al., 1984)	CITRATE /ETOH (Matsson and Rydberg, 1981)
Centrifuge cells 150g, 8 min	150g, 8 min	Centrifuge 10 min 1000 rpm	1000 rpm, 4°C 10 min
Wash in HH + culture medium + 0.037 µg/ml colchicine	--	Wash in HH	Wash in PBS
Centrifuge 150g, 10 min	--	Centrifuge 10 min 1000 rpm	1000 rpm, 4°C 10 min
Resuspend in 75 mM KCl + 0.037 µg/ml colchicine	Resuspend in 0.7 ml 75 mM KCl+20 µg/ml P	Resuspend in 75 mM KCl	Resuspend in 0.1% Na-citrate + 50 µg/ml PI at 10^6 cells/ml
4°C 7 min	25°C 10 min or 37°C 25 min	4°C 10 min	25°C 20 min
Centrifuge 200g, 10 min	--	1000 rpm, 8 min	--
Resuspend in buffer: 25 mM Tris-HCl 0.75 M hexylene glycol 0.5 mM $CaCl_2$ 1.0 mM $MgCl_2$ 0.037 µg/ml colchicine at pH 7.5	Add 0.3 ml 50 mM KCl + 20 µg/ml PI + 1.5% Triton X-100	Wash in buffer 15 mM Tris-HCl, pH 7.2, + 2 mM EDTA + 0.5 mM EGTA + 80 mM KCl + 20 mM NaCl + 14 mM β-mercaptoethanol	Add ethanol to 20% final concentration while vortexing
Incubate 37°C 10 min	25°C 4 min	Centrifuge 1000 rpm, 8 min	--
Incubate 4°C 30 min	--	Resuspend in buffer as above + 0.2 mM spermine + 0.5 mM spermidine + 0.1% digitonin	--
Shear with needle or Virtis homogenizer	Shear with needle	Vortex 10-20 s	Vortex

CITRIC ACID (Collard et al., 1980a)	TRIS/$MgCl_2$/TRITON X (Otto et al., 1980; similar procedures described by Disteche et al., 1982; Bijman, 1983)	MODIFIED PROPIDIUM IODIDE (Cram et al., 1983)	HEPES/$MgSO_4$ (chapters 6 and 7)
Centrifuge cells 100 rpm, 4°C 10 min	Centrifuge cells 1000 rpm 5 min	Centrifuge 150g, 8 min	Centrifuge 1000 rpm, 4°C 10 min
Wash twice in PBS	--	--	--
1000 rpm, 4°C 10 min	--	--	--
Resuspend in 75 mM KCl	Resuspend in 20 mM Tris-HCl, 40 mM $MgCl_2$ (+ EB and mithramycin)	Resuspend in 1.0 ml 75 mM KCl + 5 µg/ml PI	Resuspend in buffer: 50 mM KCl, 5 mM HEPES, 10 mM $MgSO_4$, 0.15 mg/ml RNase, 3 mM dithiothreitol, pH 8.0
25°C 5-30 min	4°C 10 min	25°C 10 min	37°C 10 min
1000 rpm, 4°C 10 min	--	--	--
Remove all but 0.1 ml KCl. Add cold buffer: 2% citric acid + 1 mM $MgCl_2$ + 1 mM $CaCl_2$ + 0.1 M sucrose at pH 2.4, dropwise, while vortexing; 10^7 cells/ml	Add Triton X-100 to final concentration: 0.25%	Add 0.5 ml 75 mM KCl + 0.1% Triton X-100 + 5 µg/ml PI + 1.0 mg/ml RNase	Add 1/10 volume part of 2.5% Triton X-100
--	room temperature 10 min	25°C 3 min	25°C 10 min
Shear with needle at 4°C	Disrupt with homogenizer or needle	Shear with needle	Shear with needle
Dilute with 0.1 M Tris-HCl, 0.1 M NaCl, 5 mM $MgCl_2$ + EB, pH 7.4	Dilute with 150 mM NaCl, 40 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.5	Incubate 37°C 30 min	Incubate 37°C 30 min Add 10 mM sodium citrate after staining

variability can also be expected at higher passages (Bartholdi et al., 1984). Good mitotic cell yield can be obtained with fibroblast cell cultures passaged 48 h before colchicine treatment, at a dilution such that the cultures are 50-70% confluent when the colchicine is added. Improved flow karyotype resolution has been shown for Chinese hamster cells (Otto et al., 1980) and for rat rhabdomyosarcoma cells (Kooi et al., 1984) after short-term culturing in vitro.

Various spindle poisons are employed to collect cells in mitosis: colchicine, vinblastine, and vincristine (see chapter 6). Mitotic cells can also be collected by synchronizing the cells with thymidine and then adding vinblastine for a short period when the wave of synchronized cells pass into mitosis. This has been reported to reduce variability in chromosome contraction resulting from varying exposure to the blocking agent (Collard et al., 1980a). Mitotic cells can also be discarded after a short colchicine treatment; only the mitotic cells after a second period of colchicine are collected (Cram et al., 1983). Differential trypsination of colchicine-treated cells has also been used for cell collection (Stubblefield et al., 1975).

Number of cells

The number of cells required to give optimal results with the different isolation protocols varies. Carrano and coworkers (1979b) determined that optimal flow karyotype resolution was obtained with the hexylene glycol method with a cell concentration of $5x10^6$ per ml. The Hepes/$MgSO_4$ method has the advantage that concentrations from $5x10^6$ cells/ml to as few as several thousand cells/ml result in high resolution flow karyotypes (chapters 6-9). Although studies of the effect of cell concentrations have not been presented for the other methods in table 3.2, high cell concentrations (from 10^6 to 10^8 cells/ml isolation solution) are usually reported (Matsson and Rydberg, 1981; Sillar and Young, 1981; Lalande et al., 1984; Disteche et al., 1982; Collard et al., 1980a).

Prewashing

Washing the collected cells before they are swollen is called for in several procedures (Matsson and Rydberg, 1981; Collard et al., 1980a; Lalande et al., 1984). Other protocols have been simplified for increased yield and allow direct addition of the final buffer to the pellet of mitotic cells (Aten et al., 1980; Otto et al., 1980; Cram et al., 1983; Bijman, 1983; chapter 6).

Cell swelling

All chromosome isolation methods include a step in which the chromosomes are swollen in a hypotonic buffer (usually diluted KCl), although the length of time and the temperature at which this swelling takes place vary (see table 3.2). The optimal KCl concentration has been investigated by Bijman (1983) and in chapter 6. Swelling in the citrate/ETOH procedure takes place in 0.1% citrate (Matsson and Rydberg, 1981). Cells are centrifuged after the swelling step in several methods (hexylene glycol, polyamine, and citric acid).

Disruption of cells

Triton X-100 (Aten et al.,1980; Bijman, 1983; Otto et al., 1980; Cram et al., 1983; chapter 6) and digitonin (Sillar and Young, 1981; chapter 6) are two detergents commonly used to solubilize the plasma membrane for chromosome isolation. Detergents are not used in the citric acid method (Collard et al., 1980a), the citrate/ETOH method (Matsson and Rydberg, 1981) or in the hexylene glycol method (Carrano et al., 1979b).

Cells can be physically disrupted to release chromosomes into suspension with a homogenizer (hexylene glycol method: Carrano et al., 1979b) or by shearing through a needle (hexylene glycol method: Carrano et al. (1979b), Yu et al. (1981); PI method: Aten et al. (1980), Cram et al. (1983); Tris/$MgCl_2$ method: Bijman (1983), Otto et al. (1980); Hepes/$MgSO_4$ method: chapter 6; citric acid method: Collard et al., 1980a). Rapid vortexing is recommended for the polyamine (Sillar and Young, 1981) and the citrate/ETOH (Matsson and Rydberg, 1981) procedures to reduce damage to interphase nuclei. Stöhr et al. (1982) reported the use of ultrasonication to disrupt cells.

Yield

Yield is higher in methods with few steps than in methods in which cells can be lost during centrifugation (Yu et al., 1981). Approximately 25%-75% of the number of chromosomes expected from the number of original mitotic cells are recovered using the Hepes/$MgSO_4$ method (chapter 11).

Enzyme treatments

Deoxyribonuclease (DNase) was employed by Kooi et al. (1984) before chromosome isolation to remove debris from cells isolated from tumor tissue. Trypsin added to the hexylene glycol method was reported to reduce chromosome clumping, but to increase the level of the background continuum (Carrano et al., 1979b).

Ribonuclease (RNase) added to the propidium iodide method (Cram et al., 1983) and the Hepes/$MgSO_4$ method (chapter 6) reduces fluorescent signals resulting from the binding of PI to double-stranded RNA. RNase treatment in conjunction with the hexylene glycol method has been reported to decrease clumping, but increase the amount of small debris (Carrano et al., 1979b).

Storage

Chromosomes isolated with any of the procedures in table 3.2 can be stored at least several weeks before analysis (citrate/ETOH: Matsson and Rydberg, 1981; polyamine: Sillar and Young, 1981; hexylene glycol: Carrano et al., 1979b; citric acid: Collard et al., 1981; Tris/$MgCl_2$: Kanda et al., 1983; Hepes/$MgSO_4$: chapter 6).

Staining

The DNA fluorochromes most commonly used in the flow cytometric analysis of chromosomes are propidium iodide (PI), Hoechst 33258 (HO), chromomycin A3 (CA3) and DAPI. All these dyes can be used on chromosomes isolated with the Hepes/$MgSO_4$ method (chapters 6 and 7; Meyne et al., and with the hexylene glycol method after $MgCl_2$ addition (Langlois et 1980) Chromosomes isolated using the polyamine buffer can be stained with PI (Sillar and Young, 1981), although rapid degradation of the flow karyotype with this stain has been reported by Lalande et al. (1984); with DIPI (Lebo et al., 1984); with HO, if the dye is added within 3-4 hours of measurement (Young et al., 1981; Fantes et al., 1983; Lalande et al., 1984); and with CA3, if the stock solution of CA3 contains $MgCl_2$ Lalande et al., 1985). Chromosomes isolated using the citric acid method can be stained with EB, with a combination of ethidium bromide (EB) and mithramycin, or with HO and CA3, if they are transferred first to a neutral buffer containing $MgCl_2$ (Collard et al., 1984). HO and the dye combination, EB+mithramycin, have been used to stain chromosomes isolated using the Tris/$MgCl_2$ buffer (Otto et al., 1980; Disteche et al., 1982; Kunkel et al., 1982). For the PI method, PI serves as both the stabilizer and the stain (Aten et al., 1980). However the replacement of PI with psoralen has been shown to allow HO staining using this procedure Yu et al., 1981).

Banding

Chromosomes isolated using some of the isolation procedures in table 3.2 can be fixed to slides after sorting and can be stained to produce banding patterns. Chromosomes isolated with the hexylene glycol method can be banded by quinacrine staining (Carrano et al., 1976; Carrano et al.,

1979a, b; Disteche et al., 1981). A large proportion of chromosomes isolated using the PI method can be banded using a combination of actinomycin D and DAPI (Buys et al., 1982). Recently, banding of chromosomes isolated using the polyamine buffer has been reported (Fantes et al., 1983; Lebo et al., 1984). Chromosomes isolated using the Tris/$MgCl_2$ buffer could be banded, if they were diluted during sorting in a sheath fluid containing hexylene glycol (Disteche et al., 1982). Bands can also be produced in chromosomes isolated using the Hepes/$MgSO_4$ method using a hot salt (SSC)/trypsin/Giemsa protocol. In this case, the chromosomes are fixed with a protein cross-linker before being attached to microscope slides (chapter 11).

Applicability to various cell types

Most of the chromosome isolation protocols were developed using cells from Chinese hamster fibroblast cultures. Chinese hamster cells contain approximately twelve, relatively large, chromosome types, which can be easily discriminated in most flow cytometers (Tris/$MgCl_2$: Otto et al., 1980, Bijman, 1983; polyamine buffer: Sillar and Young, 1981; hexylene glycol: Gray et al., 1975; PI method: Aten et al., 1980; modified PI method: Cram et al., 1983; citric acid: Collard et al., 1980b; Hepes/$MgSO_4$ method: chapter 6; hexylene glycol in combination with acid treatment: Stohr et al., 1982; Stubblefield et al., 1975).

Chromosomes have been isolated for flow cytometry from the mouse (2n=40) with the hexylene glycol method (Disteche et al., 1981), the Tris/$MgCl_2$ method (Disteche et al., 1982), and the Hepes/$MgSO_4$ method (chapter 12 and 16). Chromosomes have also been isolated for flow cytometry from the Indian muntjac (2n=7) (hexylene glycol: Carrano et al., 1976; citric acid: Collard et al., 1980a), the rat (2n=42) (citric acid: Collard et al., 1982; Hepes/$MgSO_4$, chapter 10, the kangaroo rat (2n=13) (hexylene glycol: Stöhr et al., 1980), and the chicken (Stubblefield and Oro, 1982).

Chromosomes have been isolated from human fibroblast cultures (2n=46) using the hexylene glycol method (Gray et al., 1979), the Hepes/$MgSO_4$ method (chapter 7; Meyne et al., 1984), the modified PI method (Meyne et al., 1984), the polyamine buffer (Sillar and Young, 1981), the Tris/$MgCl_2$ method (Kanda et al., 1983; Kunkel et al., 1982), the citric acid method (Collard et al., 1981), and with psoralen stabilization (Yu et al., 1981).

Human peripheral blood lymphocyte cultures have also served as a chromosome source using the hexylene glycol procedure (Yu et al., 1981), the citrate/ETOH method (Mattson and Rydberg, 1981), the polyamine buffer (Young et al., 1981), the Hepes/$MgSO_4$ method (chapter 7), and the PI method with psoralen stabilization (Yu et al., 1981).

Chromosome suspensions have been prepared in several laboratories from human lymphoblastoid suspension cell cultures using the polyamine buffer (Sillar and Young, 1981; Lalande et al., 1984, 1985; Lebo et al., 1984; Krumlauf et al., 1982; Wirchubsky et al., 1983; Bernheim et al., 1984).

Chromosomes can also be isolated from the low number of cells found in clinical amniotic cell cultures using the Hepes/$MgSO_4$ method (chapter 9).

Using the PI method, chromosomes could be isolated from trypsinized rat rhabdomyosarcoma tumor tissue after vincristine treatment in vivo and from in vitro cell lines of this tumor (Kooi et al., 1984).

Protein preservation

The pattern of isolated proteins on a polyacrylamide gel has been used to show that proteins are better preserved in chromosomes isolated in the presence of Mg^{++} ions than in chromosomes isolated in the presence of polyamines (Adolph, 1980). The relative histone composition of chromosomes has also been shown to be maintained in the Hepes/$MgSO_4$ procedure by antibody labeling and HPLC analysis of the proteins extracted from sorted chromosomes (chapter 12).

Additional applications

Hybridization of DNA sequences to chromosomes sorted directly onto nitrocellulose filters has been shown for chromosomes isolated using the polyamine buffer (Bernheim et al., 1983; Lebo et al., 1984), the Hepes/$MgSO_4$ buffer (chapter 15), and the citric acid buffer (Collard et al., 1985).

Slit scan measurements have been performed with chromosomes isolated using the PI method after additional stretching at 37°C for 30 min (Lucas et al., 1983).

Molecular weight of DNA

In a search for methods that preserve the DNA for cloning long DNA sequences, the size of the DNA fragments that can be isolated from chromosomes prepared using various methods has been reported. DNA fragments with a molecular weight of approximately $30x10^6$ Dalton have been isolated from chromosomes prepared with the psoralen method and the hexylene glycol method (Yu et al., 1981). High pH in the latter method has been recommended to preserve high molecular weight DNA (Wray and Stubblefield, 1977). Chromosomes sorted using the high speed sorter in Livermore also can be used as a source of $10\text{-}100x10^6$ Dalton DNA fragments (Gray et al., 1981). The molecular weight of DNA extracted from chromosomes isolated in the polyamine buffer has been shown to be significantly larger than that from chromosomes isolated in the hexylene

glycol buffer ($200x10^6$ vs. $2x10^6$ Dalton) (Blumenthal et al. 1979). This difference was ascribed to nuclease inhibition by the chelators in the polyamine buffer. Micrococcal nuclease digestion has been used to demonstrate that nucleosome structure is better preserved in polyamine buffer than in hexylene glycol buffer (Tien Kuo, 1982). DNA fragments from chromosomes, isolated in citric acid buffer and later suspended in a neutral pH buffer containing 5 mM $MgCl_2$, have been reported to be 60-100 kbase pairs ($40-64x10^6$ Dalton) in size (Collard et al., 1984). A comparison of the molecular weight of DNA fragments from chromosomes isolated using several procedures, including the Hepes/$MgSO_4$ procedure, shows that the DNA fragments extracted from the chromosomes in all cases are larger than 50 kbase pairs in size (chapter 11).

Chapter 4

FLOW CYTOMETERS

The fundamental principle of flow cytometry is that cells or cell fragments are made to flow in suspension through an intense light source. Here, optical signals, which contain biochemical or morphological information about the particles, are generated and can be quantified.

Flow cytometers made their appearance in the late sixties and were used primarily for the analysis and separation of cells on the basis of their size, DNA content and surface antigens (Fulwyler, 1965; Kamentsky et al., 1965; Van Dilla et al., 1969; Göhde and Dittrich, 1970; Hulett et al., 1969; Kamentsky and Melamed, 1967; Steinkamp et al., 1973; Bonner et al., 1972).

For chromosome analysis, the flow cytometers must be capable of sensitive and precise measurements. The DNA content of chromosomes, and the amount of fluorescent DNA stain that can be bound to them, is small compared to that of cells. The various chromosomes of a given species differ only slightly in DNA content. However, the DNA content of a given chromosome type in the cells of an individual is remarkably constant (Mayall et al., 1984). Efforts to improve the sensitivity and precision of flow cytometric measurements through technical changes have paid off in better chromosome resolution. This in turn has facilitated karyotype analysis and the collection of pure chromosome fractions. The technical facets in the technique of flow cytometry are discussed in this chapter.

Single laser flow cytometry

The basic features of a single laser flow cytometer are illustrated in figure 4.1. An example of a single laser machine, in use in many laboratories, is the FACS II (Fluorescence Activated Cell Sorter, Becton and Dickinson, Sunnyvale, CA) (Fulwyler, 1979; Sweet, 1979).

The sample, equilibrated in fluorescent dye, is injected slowly into a faster-flowing stream of fluid. Since minimal mixing of the sheath fluid and the sample occurs during analysis, a sheath fluid of water is adequate. However, if chromosomes or nuclei are to be sorted, a stabilizing agent is usually added to the sheath fluid to keep chromosomes intact during and after sorting (Lalande et al., 1984; Collard et al., 1984; Disteche et al., 1982). The sheath fluid provides a laminar sheath, surrounding and

aligning the particles by hydrodynamic focusing. The chromosomes follow almost identical trajectories and flow at a high rate through the laser beam. The laminar flow also serves to separate chromosomes widely from each other so that they pass one-by-one through the laser spot. In the FACS, the intersection of the laser beam and the sample stream is in air, after the stream has passed through the 50-100 μm orifice of the nozzle. In other systems, this intersection takes place in the narrow channel (100-250 μm) of a square quartz cuvette.

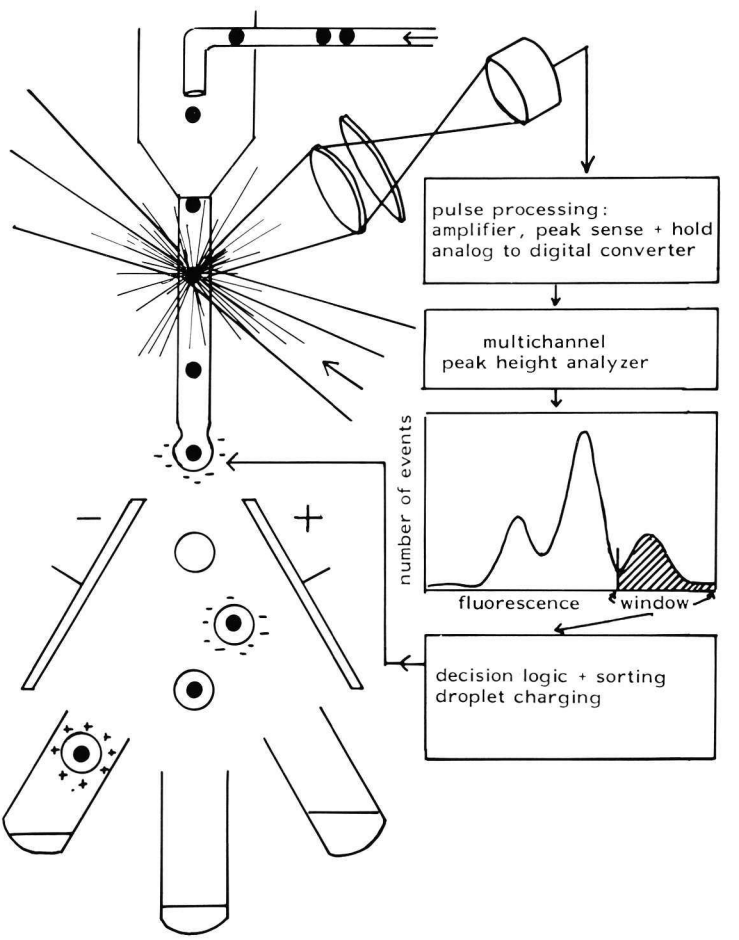


Figure 4.1. The basic principles of a single laser flow cytometer (see text).

Detection of optical signals

The chromosomes are in the laser beam for 1-5 μs. They scatter light, and any fluorescent molecules they contain will fluoresce if excited by the wavelength of the laser being used. Light scattered by the chromosomes in a forward direction (FLS, 0-13° from the axis of the laser beam) can be collected and converted by a photodiode into an electric signal. A narrow blocking bar prevents the incident laser beam from directly entering the FLS detection system. Forward light scatter intensity has been shown to be a good measure of the cross-sectional area of spherical particles like cells (Steinkamp et al., 1973; Van den Engh et al., 1979; Van den Engh et al., manuscript in prep.). FLS has also been used to discriminate between debris and chromosomes (Bernheim et al., 1983; Sillar and Young, 1981; Lalande et al., 1984; Collard et al., 1984).

A microscope objective, arranged with its optical axis orthogonal to both the laser illumination beam and the direction of sample flow, is the first step in the main collection optics. Light collected through this objective contains information on the scatter (light with the same wavelength as the incident laser light) and the fluorescence of the particles. With a combination of mirrors, colored glass filters, and interference filters, these optical signals can be detected on separate photomultipliers.

It is possible to measure at least two fluorescent properties and one light scatter property for each cell or cell fragment in the FACS. If more than one fluorescent stain is to be used, the single laser dictates that they be excited with the same wavelength and that their emission spectra be spectrally separable. Loken (1980), however, has described the measurements of two fluorescent dyes, which did not meet these criteria, using a single laser emitting multiple wavelengths of light.

Signal processing

The optical signals must be translated into electric signals that can be displayed or stored. Each photomultipler or photodiode produces an electric current proportional to the intensity of the optical signal it detects. Each pulse is amplified and can be visualized on an oscilloscope. If desired, the area of each pulse can be determined with an integrating amplifier. The logarithm of these values can also be determined. "Peak sense and hold" circuitry (PSH) determines the peak of the resultant pulse and holds this value temporarily. If two properties have been measured for each particle, their values can be correlated and shown on a two-dimensional oscilloscope to produce a dot plot. Each dot in this type of plot represents the measurements of a single particle, and clusters indicate subpopulations with similar optical properties.

The peak value from the PSH is digitized in an analog-to-digital converter (ADC), before being stored in the memory of a multichannel pulse height analyzer (MPHA). A univariate frequency distribution of the fluorescence or scatter intensity of the particles can be constructed in the 256-channel memory of a MPHA. For bivariate plots, the digital values of two parameters for each particle are accumulated in a 64 by 64 matrix of a MPHA. Interfacing to the computer allows transmission of the distribution from the MPHA for storage and later analysis.

Sorting

Particles whose peak values held in the PSH fall within ranges set by the experimenter can be sorted. For sorting, the liquid jet is vibrated with the help of a piezo-electric crystal at a frequency such that droplets (which contain individual particles) break from the sample/sheath stream in a regular manner at a fixed distance below the measuring point (Fulwyler, 1965; Sweet, 1979). Sort logic circuitry compares the signals of each particle with preset thresholds ("windows"), which specify upper and lower limits for the optical parameter(s) being measured. Complexly-shaped windows can be set using computer based channel analyzers that display three-dimensional dual parameter histograms (Peters et al., 1982). The fluid stream is given an electric charge just before the droplet containing the desired particle breaks from the stream. After droplet formation, the fluid stream is brought back to ground potential. The droplets then pass through a constant transverse electric field; charged particles are deflected into collection tubes. In the FACS, particles can be sorted at a rate of approximately 1000 per second. In the high speed sorter developed in Livermore, cells or chromosomes can be sorted at rates in excess of 20,000 per second (Peters et al., 1985).

Multi-beam flow cytometry

The most important technological advancement for chromosome analysis has been the dual laser flow cytometer. This system allows greater freedom of choice in dyes than the single laser machine. The excitation spectra of the dyes can be different, and their emission spectra may overlap.

Dual laser machines have been developed in several laboratories (Stöhr et al., 1977; Shapiro et al., 1977; Arndt-Jovin et al., 1980; Steinkamp et al., 1979; Dean and Pinkel, 1981; Steinkamp et al., 1982; Lebo and Bastian, 1982; Peters et al., 1985) and are available commercially from several firms. A three-laser flow cytometer (3-FC), specifically designed for chromosome analysis, is also in development (Van den Engh et al., manuscript in preparation).

The optical arrangement of the Livermore dual beam cytometer (DBC) (Dean and Pinkel, 1981), on which many of the experiments described in this thesis were performed, is shown in figure 4.2. The optical arrangement of the 3-FC is shown in figure 4.3. These machines differ from the FACS in that the intersection of the sample stream with the two laser beams occurs within a quartz cuvette. Fluorescent particles pass sequentially through two spatially separated laser beams tuned to emit light with different wavelengths. The two beams intersect the sample stream at spots approximately 200 μm apart. A given particle takes approximately 20 μs to transverse both beams.

Various arrangements of the laser-focusing and -shaping lenses are used in multi-laser machines. In the DBC, cylindrical and cut-off spherical lenses shape each laser spot into an ellipse. A typical light spot measures 20 x 100 μm. The beams can be independently focused on the sample stream (figure 4.2A; Dean and Pinkel, 1981; Steinkamp et al., 1979; Watson, 1981). In modifications of the FACS for dual beam measurements (Lebo and Bastian, 1982; Visser, personal communication), the two beams can be directed with mirrors and shaped with cut-off spherical lenses (De Josselin-de Jong et al., 1984). In the illumination arrangement of the 3-FC, the beam shaping optics allow independent focusing of three laser beams, and there is ample space for the lenses (figure 4.3A).

Detection optics

The optical signals arising from particles in the two beams are collected by a spherical lens and focused on two pinholes in the DBC and the 3-FC. The light from one beam goes directly to a detection assembly. The light from the second beam is reflected off a mirror to a second set of detection optics (figures 4.2C; 4.3C). This spatial separation allows the fluorescence originating at each of the two beams to be detected on a separate photomultiplier. In other dual laser systems, the separation of the signals originating at the two excitation beams is based mainly on chromatic filtering (Steinkamp et al., 1979; Steinkamp et al., 1982). The signals from the third beam in the 3-FC are reflected off of a second mirror and are detected in a similar set of filters and photomultipiers (not shown in figure 4.3).

A combination of beam splitters, filters, and photomultipliers like that found in a single laser machine permits the measurements of at least two optical signals from the interaction of particles with each beam (figure 4.2D). In the 3-FC, two fluorescence signals and one perpendicular light scatter signal can be measured from the interaction of particles with one of the beams. The light reflected from the surfaces of the filters in front of two photomultipliers is collected and measured on a third

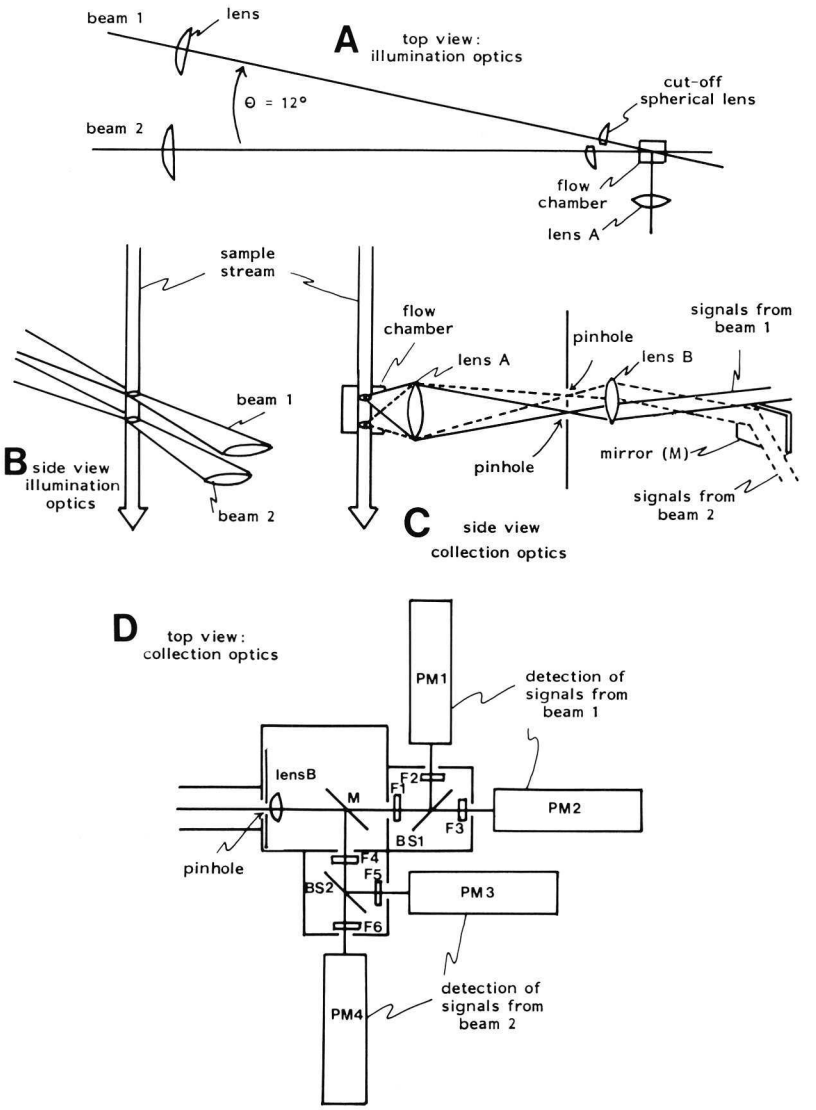


Figure 4.2. A schematic representation of the optical arrangement of a dual beam flow cytometer (redrawn from Dean and Pinkel, 1978). A) Illumination optics for two laser excitation. B) The shape of the laser spots on the sample stream. C) The collection optics, which allow spatial separation of the fluorescence signals originating from the two beams. D) The detector assembly: two photomultipliers are used to detect the signals from each beam. The light signals from beam 1 pass through filter F1, are split by the beam splitter BS 1, and pass through filter F2 or F3. The light from beam 2 is reflected by a cut-off mirror (M1) onto photomultipliers PM 3 and PM 4. The light from beam 2 passes through filters F4, and F5 or F6, and is split by the beam splitter BS 2.

photomultiplier (figure 4.3C). This detector is used to quantify the perpendicular light scatter signals, which, for cells, are usually quite strong (Van den Engh and Visser, manuscript in preparation).

Signal processing

The optical signals originating from the interaction of a given particle with the two beams in a dual beam flow cytometer are separated in time. Electronic circuitry controls the correlation and storage of these values. An amplified signal rising above an adjustable threshold at the first measuring station triggers a PSH, as in the single laser system. The value is held approximately 20 μs until the same particle has interacted with the second laser. The pulse from the first laser triggers the acceptance of fluorescent signals at the second laser during the time in which the particle is expected to be in the measuring point. The timing and the duration of the gate opening can be adjusted manually (Hiebert et al., 1981; Steinkamp and Hiebert, 1982). The signal detected at the second laser in this time period is also amplified, and the peak value is determined with a PSH. The paired values for each particle are digitized in ADC's and stored in the 2-dimensional memory of a MPHA (Peters et al., 1982; Dean and Pinkel, 1981). Alternatively, the fluorescence intensity values for each particle can be stored directly in computer memory, in what is termed listmode (Steinkamp and Hiebert, 1982; Shapiro et al., 1977; Shapiro, 1983). After the values have been stored, a "clear signal" is issued, which releases the PSH to accept a new signal at the first station. The "dead time" between the detection of a particle at the first laser and the completed storage of its fluorescent signals can be substantial (100-200 μs (Steinkamp and Hiebert, 1982).

In novel circuitry designed by Van den Engh and Stokdijk (manuscr. in preparation) for the 3-FC, this dead time has been reduced to 5 μs. In this system, particles pass the second and third lasers 20 and 40 μs after they pass the first. The optical parameters of up to 16 particles can be measured and retained at the first beam before the first particle in the series has even passed the third. A digital gate controller and a gate generator keep track of the fluorescence values that belong to each particle. In this system, not the transit time, but the speed of signal processing, becomes the limiting factor. Improvements in the speed of the circuitry make it possible to process up to eight parameters for each particle at a rate of 200 KHz.

In the 3-FC, the appearance of a particle at the first measuring point causes the gate controller and gate generator to open gates lasting approximately 5 μs for the acceptance of optical signals at each measuring point when the particle will be present. For each of eight parameters that

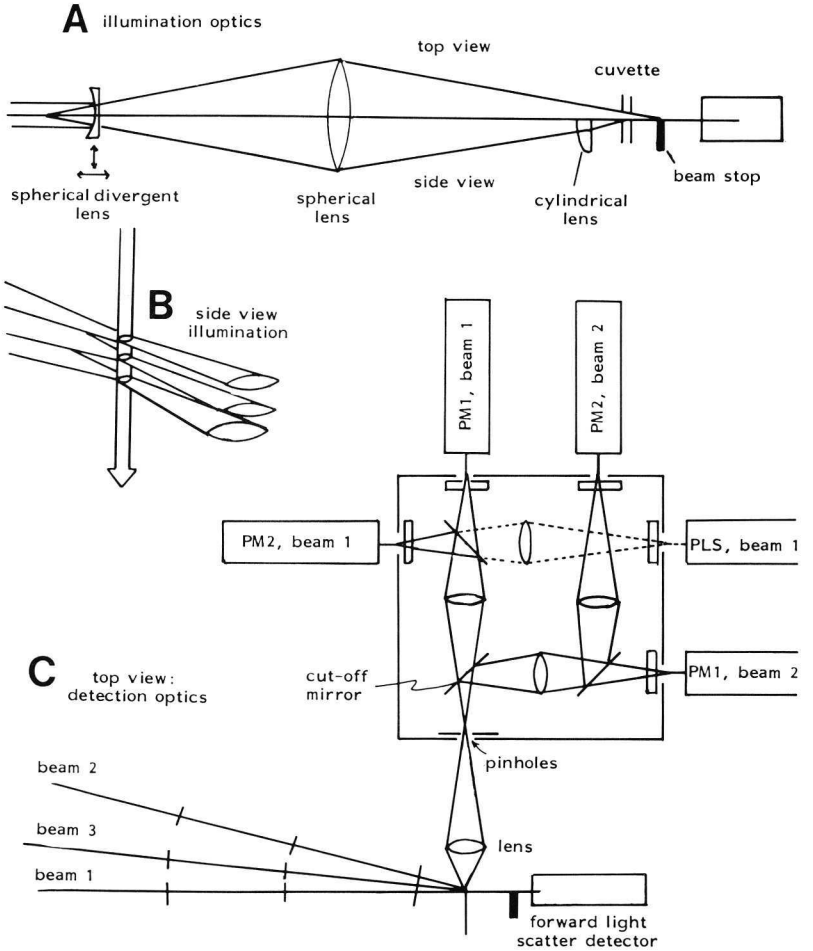


Figure 4.3. A schematic representation of the optical arrangement of a 3-laser flow cytometer developed for chromosome analysis (3-FC, van den Engh et al., manuscript in preparation). A) Optical system allowing focusing, steering and shaping of each of three lasers beams into an elliptical shape at the flow cell. B) Side view, indicating the shape of the laser beams at sample stream. C) Top view of the detector assembly: fluorescence signals from each beam are imaged on a pinhole and focused by lenses onto photomultipliers. Signals from beam 2 are reflected off a mirror and focused on two photomultipliers (PM 1 and PM 2, Beam 2) after passing a beam splitter and filters. Signals from beam 1 pass over the mirror and are focused on two other photomultipliers (PM 1 and PM 2, beam 1). In this system, light reflected off of the filters in front of the photomultipliers for beam 1 is also collected and is focused on another photomultiplier (PLS, Beam 1). Perpendicular light scatter (PLS), a relatively strong signal, can be measured here. This allows measurement of two fluorescence signals and one scatter signal (in addition to forward light scatter) at beam 1.

can be measured for each cell, the detected signal triggers a PSH. The peak value of the pulse is passed to a sample hold circuit. In this way, the PSH is free to accept a new cell before the previous one has been digitized. An ADC accepts the analog signal from the sample hold, and, in a period of 5 μs, converts the signal to its digital value. The ADC for each of the 8 parameters puts its value into a buffer and is also free to work on the next value given to it by the sample hold. Each ADC has 16 buffers, which it fills sequentially. When flags on the buffers of all 8 ADC's indicate that all 8 parameters for a given particle have been collected, a bus controller arranges the transfer of the eight values to a Hewlett-Packard 200 series computer for list mode storage. Periodically, a marker indicating elapsed time is placed among the list of data. The speed of this electronic system should make it ideal for kinetic measurements and for the high speed analysis and sorting of cells or chromosomes.

Data display

The data measured for a population of particles can be displayed in several ways 1) univariate frequency distribution with the fluorescence or scatter intensity in channel numbers on the abscissa, and the number of events on the ordinate, 2) in a dot plot, in which each dot represents the coordinates of one cell in a bivariate distribution, where X and Y are the intensities of optical signals, or 3) bivariate frequency distributions in which the number of events, with optical signals indicated by the X,Y coordinate, is indicated by the height of the Z-axis by contour lines or gray value gradations, or in an isometric plot.

Technological improvements for increased measurement sensitivity and resolution

In both single and dual laser machines, improvements in the sensitivity and precision of the measurement can improve the quantification of dimly stained particles and can increase the resolution of differently stained subpopulations in a suspension. The sensitivity can be increased by improving the signal-to-noise ratio. Higher precision and resolution can be achieved by increasing the number of photoelectrons detected for each particle. This reduces the photon-statistical error in the measurement.

Improvements in the illumination, flow characteristics, and collection optics and in the fluorescence characteristics of the stains used can increase the sensitivity and precision of flow cytometric measurements. However, if the inherent variability in the particles being analyzed is high, or if inhomogeneous staining occurs, these factors will limit flow

cytometric resolution. Improvements in the machine itself may then have little noticeable effect.

Illumination

For optimal measurement, the product of the molar absorbivity of the dye being used and the intensity of the light available at a given wavelength should be maximized (Jensen et al., 1977). On this line, improvements in flow karyotype resolution have been reported when high light output lasers are used (Lebo and Bastian, 1982; Bartholdi et al., 1983). Alternatively, the illumination intensity can be increased by decreasing the size of the beam spot (Shapiro, 1983; Shapiro et al., 1983; Lalande et al, 1984; 1985). A smaller spot size may also improve sensitivity by reducing the amount of laser light scattered by the sample stream, which may reach the collection optics. The elliptical beam spot size in the DBC is approximately 20 x 150 μm, and in the 3-FC it is approximately 10 x 50 μm. Bartholdi et al. (1983) reported improved karyotype resolution with the high illumination intensities (4 MW/cm^2) achieved with a spot size of 2 x 27 μm. If the beam spot becomes too small, however, alignment becomes difficult.

If the width of the beam is less than the length of the particles being measured, peak height is an unreliable measurement of total fluorescence. Particles with the same total fluorescence, but with different lengths, will produce pulses of differing durations, and therefore, with different heights. Integrating amplifiers must be used to produce a signal proportional to the total fluorescence. If the width of the beam is at least 2-3 times greater than the length of the particles, the peak height is approximately proportional to the integral of the pulse, or the total fluorescence of the particle.

In a slit-scan flow cytometer (Gray et al., 1979c; Lucas et al., 1983), the beam width is reduced to 2 μm. The fluorescence intensity of chromosomes, as they pass lengthwise through this spot, are sampled at 20 ns intervals. A profile of the fluorescence intensity of a chromosome, which shows a dip representing the centromere, can be produced.

Flow characteristics

The optical measurement is made in the FACS in the fluid stream in air. Stray scatter light from the laser on the stream can be reduced by measuring in a square quartz cuvette (as in the DBC and 3-FC). This can increase the ratio between the specific signal of dimly fluorescent particles and background "noise" signals. In addition, the flow rate in a

cuvette can be slower than in air, allowing more photons to be collected from each passing particle.

Flow stability ensures that all particles will pass the laser spot and the focal point of the collection optics at the same position and with the same velocity. This increases the precision of the measurement by ensuring that the illumination received by all particles is identical and that the fluorescence detected for all identical particles is the same. Higher resolution can usually be achieved at lower flow rates (150-200 events/s) using syringe-driven sample injection (Shapiro, 1983; Bartholdi et al., 1983; Lalande et al., 1984). The chromosome measurements presented in this thesis were performed in this way. A sample injection system using double sheath flow layers has also been used to improve flow stability (Bartholdi et al., 1983).

Collection optics

Optimal alignment of the focused laser spot on the sample stream and of this intersection on the focal plane of the collection optics is required for high resolution (see Dean, 1985). The sensitivity of the measurement is also increased if an image of each stream and laser beam intersection is formed at some point in the collection optics. An aperture or pinhole at this point helps to reduce fluorescence signals from the second laser beam or stray light from reaching the detector (Dean and Pinkel, 1978; Shapiro, 1983; Shapiro et al., 1983; Van den Engh, manuscr. in prep.).

After this spatial filtering, the fluorescence signals can be separated spectrally with filters. Filters are chosen for their ability to block scattered laser light, to maximally transmit the wavelengths in the fluorescence spectrum of the stain being used, and fluoresce minimally themselves. Photomultipliers can also be chosen for optimal wavelength sensitivity.

Chapter 5

FLUOROCHROMES FOR CHROMATIN ANALYSIS

A variety of fluorescent dyes are available to probe the amount, the base composition and the conformation of DNA in chromosomes and nuclei. Fluorescent molecules that can be attached to protein molecules make a wide range of antibodies available for the flow cytometric study of other properties of chromatin.

The choice of a good dye for flow cytometry is dictated by several factors. Excitation wavelengths of sufficient power must be available within the fluorescence excitation spectrum of the dye. In addition, the fluorescence emission spectra of dyes excited simultaneously in a single laser machine must be separable with optical filters.

The higher the number of photons emitted by each particle is, the higher the resolution will be that can be obtained in fluorescence distributions (Jensen et al., 1977). A good fluorescent dye, therefore, has a high quantum yield (the fraction of excited molecules that decay by fluorescence) when bound to its substrate and a low quantum yield when it is free. Because of this change in quantum yield, the bound fluorescent molecules can be quantified while the chromosomes or nuclei are uniformly suspended in equilibrium with dye in solution. The contribution of free dye to the fluorescence measurement is negligible for a good dye.

The binding of an ideal DNA dye shows high DNA specificity and stoichiometry. The relative fluorescence intensities of various chromosomes differ when different DNA dyes are used (Jensen et al., 1977; Langlois et al., 1980; chapter 6; Collard et al., 1984). These differences reflect the differences in base pair specificity of the dyes (Langlois et al., 1980). Some stains are specific for certain base pairs in DNA. Hoechst 33258 (HO) and chromomycin A3 (CA3) are prime examples. These dyes have proven particularly useful as complementary stains in dual beam flow cytometry, for the resolution of a large number of human chromosome types (Gray et al., 1979b). Because the fluorescence emission spectrum of HO overlaps the fluorescence excitation spectrum of CA3, efficient energy transfer can occur when the dyes come in close proximity in the DNA (Langlois et al., 1980; Langlois and Jensen, 1979; chapter 8). This dye combination is one of several, which through energy transfer, give

enhanced banding patterns on metaphase chromosomes on slides (Sahar and Latt, 1980; reviewed by Schweizer, 1981; Latt et al., 1980).

In this chapter, a selected group of the many available fluorescent biological dyes is discussed. Various optical properties of these dyes are indicated in table 5.1. Other fluorescent dyes are reviewed by Latt (1979) and Crissman et al. (1979). The fluorescence and absorption spectra of many dyes can be found in Porro et al. (1963) and Porro and Morse (1965).

Ethidium bromide and propidium iodide

Ethidium bromide (2,7-diamino-9-phenyl-10-ethyl phenanthridinium bromide) (EB) was the first fluorochrome to be used in the flow analysis of chromosomes (Gray et al., 1975b; Stubblefield et al., 1975). EB and its analogue, propidium iodide (PI, 3,8-diamino-5-diethylmethylaminopropyl-6-phenyl phenanthridinium diiodide), are used as dyes for the measurement of the total DNA content in chromosomes and nuclei, after RNA is digested with RNase. Neither the binding nor the fluorescence quantum yield is dependent on the base composition of DNA. The structures of EB and PI are shown in figure 5.1. Both EB and PI bind primarily by intercalating in double-stranded DNA and RNA (LePecq and Paoletti, 1967; Hudson et al., 1969; Waring, 1970). Intercalation of each EB or PI molecule has the effect of adding one base pair to the DNA helix (LePecq, 1971) and causes the unwinding of closed circular DNA (Waring, 1970; Wang, 1974; see Darzynkiewicz, 1984). Figure 5.2 shows the effect of an intercalating dye

Table 5.1

ABSORPTION AND FLUORESCENCE CHARACTERISTICS OF DNA STAINS

stain	bound to DNA or free	absorption Xmax (nm)	absorption e ($M^{-1}.cm^{-1}x10^{-3}$)	fluorescence Xmax (nm)	fluorescence X(quantum yield)
EB	-DNA	480[a]	5.2[b]	645[a]	0.02[b]
EB	+DNA	520[a]	2.9[b]	620[a]	0.26[c]
HO	-DNA	338[d]	42[e]	505[d]	0.01[e]
HO	+DNA	356[d]	29[e]	465[d]	0.6[e]
DAPI	-DNA	340[f]	27[f]	453[f]	0.05[f]
DAPI	+DNA	347[f]	24[f]	458[f]	0.9[f]
CA	-DNA		+/-1[g]		0.01[g]
CA	+DNA	430[g]	6.2[h]	570[g]	0.05[c]

[a] LePecq and Paoletti, 1967.
[b] Angerer et al., 1974.
[c] Langlois and Jensen, 1979.
[d] Latt and Wohlleb, 1975.
[e] Latt and Stetten, 1976.
[f] Lin et al., 1977.
[g] Jensen et al., 1977.
[h] Jensen, 1977

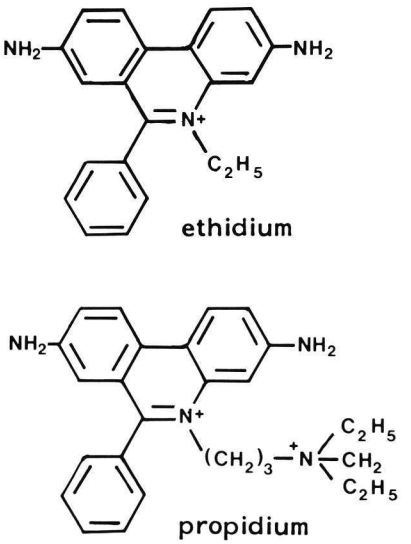


Figure 5.1. Structural formula of ethidium bromide and propidium iodide (redrawn from Latt, 1979).

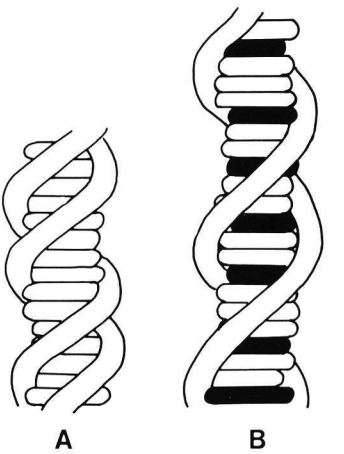


Figure 5.2. Schematic illustration of the lengthening and unwinding of the DNA double helix caused by intercalative binding of ligands such as propidium iodide. A) the original DNA helix, B) the helix after intercalating ligands have inserted (redrawn with permission from Cantor and Schimmel, 1980).

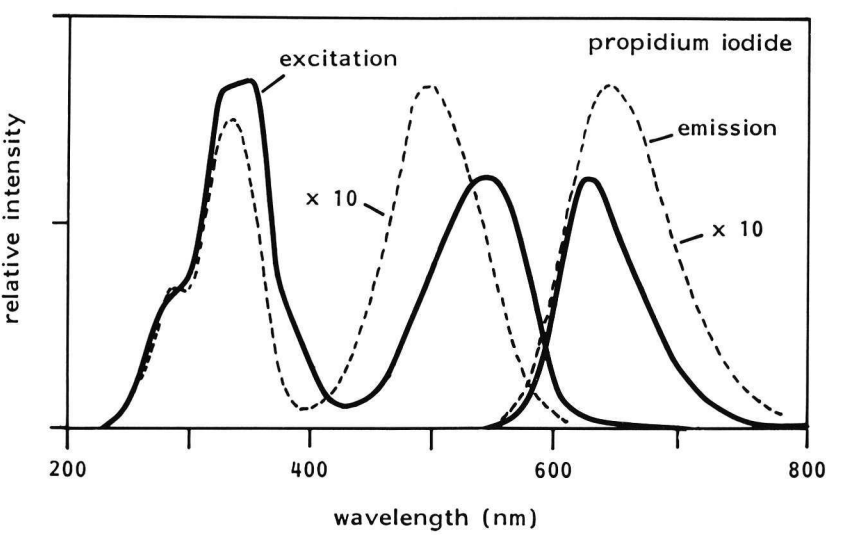


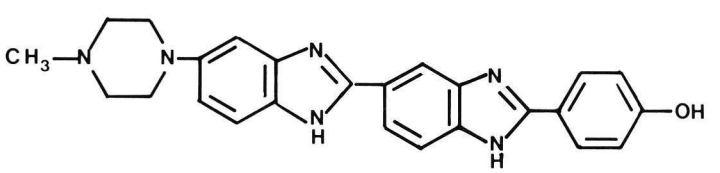
Figure 5.3. The excitation and fluorescence spectra of propidium iodide unbound (----) and complexed to DNA (——) (redrawn from Crissman et al., 1979).

on the structure of the DNA helix. EB and PI bind strongly to DNA (K_{assoc} = $2x10^6$ l/mol (Aktipis and Kindelis, 1973)). However, proteins in chromatin limit the access of these dyes to the DNA helix (Angerer and Moudrianikis, 1972; Brodie et al., 1975; Darzynkiewicz et al., 1984). The K_{assoc} of the binding of EB is 3 to 4 times lower in chromatin than in DNA (Langlois et al., 1980). A secondary binding mode, encountered only at high dye/DNA ratios, is the external electrostatic association of cationic dye molecules to $PO4^=$ groups (Aktipis and Kindelis, 1973; Le Pecq and Paoletti, 1967).

Upon binding to DNA, the fluorescence efficiency of EB is enhanced approximately 20-fold (Le Pecq and Paoletti, 1967). The fluorescence excitation and emission spectra of PI are shown in figure 5.3. The spectra of EB are similar. The fluorescence spectra of the dyes when bound to chromatin are similar to those when they are bound to DNA (Langlois and Jensen, 1979).

Hoechst 33258

Hoechst 33258 (2-(20(4-hydroxyphenyl)-6-benzimidazolyl)-6-(1-methyl-4-piperazyl)benzimidazole) (HO) (figure 5.4) is one of a group of bisbenzimidazole dyes used to stain the DNA of cells and chromosomes. Living cells can be stained with some of these dyes (HO 33342) (Arndt-Jovin and Jovin, 1977). HO binds only weakly to RNA (Latt, 1979). Two modes for the binding of HO to DNA have been described. The primary binding mode is highly specific for adenine-thymidine (A-T) base pairs in DNA and is non-intercalative (Latt and Wohlleb, 1975). HO presumably binds externally along the major groove in the double helix (Latt, 1973). A secondary, presumably electrostatic, binding mode has been described at high dye/phosphate ratios and at very low ionic strengths. The fluorescence quantum efficiency of HO molecules bound in this way is low, however (Latt and Wohlleb, 1975; Latt and Stetten, 1976). Only 50-60% of the available HO binding sites in DNA are accessible in chromatin (Latt and Wohlleb,



Hoechst 33258

Figure 5.4. Structural formula of Hoechst 33258 (redrawn from Latt, 1979).

1975). Removal of nuclear proteins with trypsin (Brodie et al., 1975) or acid (Darzynkiewicz et al., 1984) increases dye accessability.

The spectra of HO are shown in figure 5.5. Free HO has only weak fluorescence (Latt and Wohlleb, 1975; Weisblaum and Haenssler, 1974). The fluorescence intensity of HO increases 60-70 fold when it binds to DNA (Latt and Wohlleb, 1975; Weisblaum and Haenssler, 1974). Intense fluorescence in the heterochromatin regions of mouse chromosomes has been observed (Hilwig and Gropp, 1972); these regions are known to be A-T-rich. The fluorescence emission spectra of HO bound to chromosomes and to DNA are similar (Langlois and Jensen, 1979). The fluorescence of HO is pH dependent; a pH of 7.5 results in optimal DNA specificity (Latt and Wohlleb, 1975).

HO is strikingly quenched in regions where bromodeoxyuridine (BrdU) has been incorporated in DNA (Latt, 1973). BrdU is a thymidine analogue and can be incorporated in its place during DNA synthesis. This quenching has been ascribed to resonance energy transfer when BrdU and HO come into close proximity in the major groove of the DNA helix (Galley and Purkey, 1972). This phenomenon has been used to study DNA synthesis using flow cytometry (Latt et al., 1977) and sister chromatid exchange in chromosomes on slides (Latt, 1973).

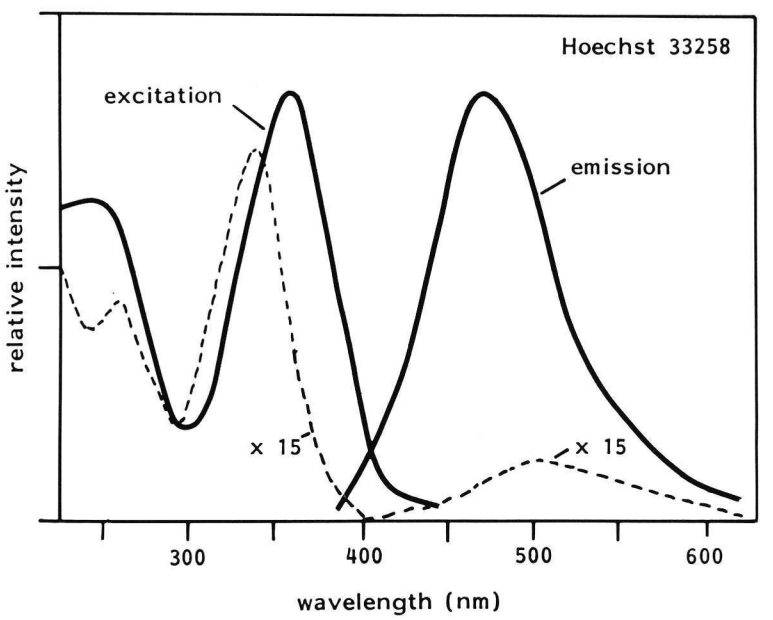
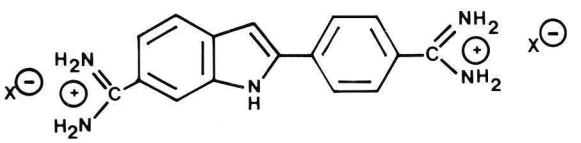


Figure 5.5. The excitation and fluorescence spectra of Hoechst 33258 unbound (----) and complexed to DNA (——) (redrawn from Latt and Wohlleb, 1975).

DAPI AND DIPI

The absorption and fluorescence spectra of the DNA stains, DAPI (4'-6-diamidino-2-phenylindole) and its analogue DIPI (4'-6-bis (2'-imidazolinyl-4H,5H)-2-phenylindole), are similar to those of HO (Lin et al., 1977). The structure of DAPI and its spectra are shown in figures 5.6 and 5.7. The binding of DAPI to DNA is specific for A-T base pairs (Lin et al., 1977). Evidence has been presented to suggest that its binding, in contrast to that of HO, is intercalative (Kapuscinsky and Skoczylas, 1978). The fluorescence intensity of DAPI increases 20- to 25-fold upon binding to DNA (Lin et al., 1977). Bright staining of certain human chromosomes, such as the 9, 15 and the Y, with these dyes has been used for their identification on slides (Schnedl et al., 1981; Schweizer et al., 1978) and in dual beam flow cytometry (Lebo et al., 1984; Meyne et al., 1984; Lalande et al., 1985).



DAPI

Figure 5.6. Structural formula of DAPI (redrawn from Lin et al., 1977).

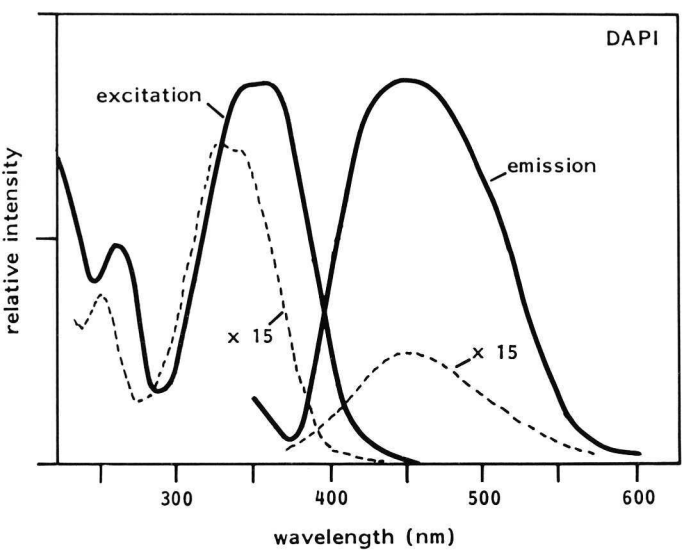
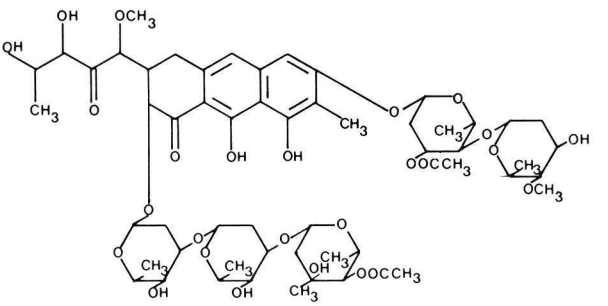


Figure 5.7. The excitation and fluorescence spectra of DAPI unbound (----) and complexed to DNA (——) (redrawn from Lin et al., 1977).

Chromomycinone dyes

The fluorescent chromomycinone antibiotics, chromomycin A3 (CA3) and mithramycin, bind specifically to DNA without intercalating (Ward et al., 1965; Waring, 1970). The structure of chromomycin and its fluorescence absorption and excitation spectra are shown in figures 5.8 and 5.9. Magenesium ions are essential for DNA staining with this group of dyes. The dye molecule interacts first with Mg^{++} ions before the complex binds to DNA (Ward et al., 1965; Jensen, 1977; Crissman et al., 1978). Although the quantum yield of CA3 is low (0.05 when bound to DNA and 0.01



chromomycin A3

Figure 5.8. Structural formula of chromomycin A3.

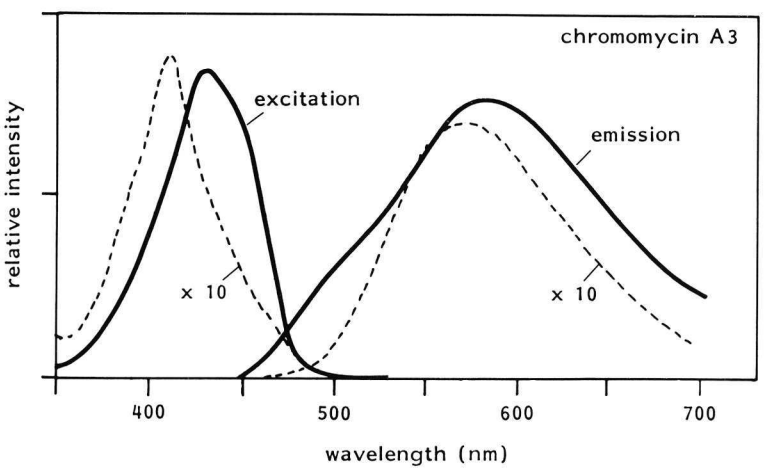


Figure 5.9. The excitation and fluorescence spectra of chromomycin A3 unbound (----) and complexed to DNA (——) (redrawn from Jensen, 1977).

when free (Jensen, 1977)), its specificity for guanine-cytosine base pairs (Ward et al., 1965; Behr et al., 1969) makes it a useful fluorophore in flow cytometry (Jensen, 1977; Crissman and Tobey, 1974).

Others

Various other stains are used in the analysis of cellular DNA content with flow cytometry. These include acridine orange, an intercalating dye whose fluorescent characteristics change with DNA conformation (Darzynkiewicz, 1979); 7-aminoactinomycin D (Zelenin et al., 1984); fluorescent anthracyclines, such as daunomycin and adriamycin (Sonneveld

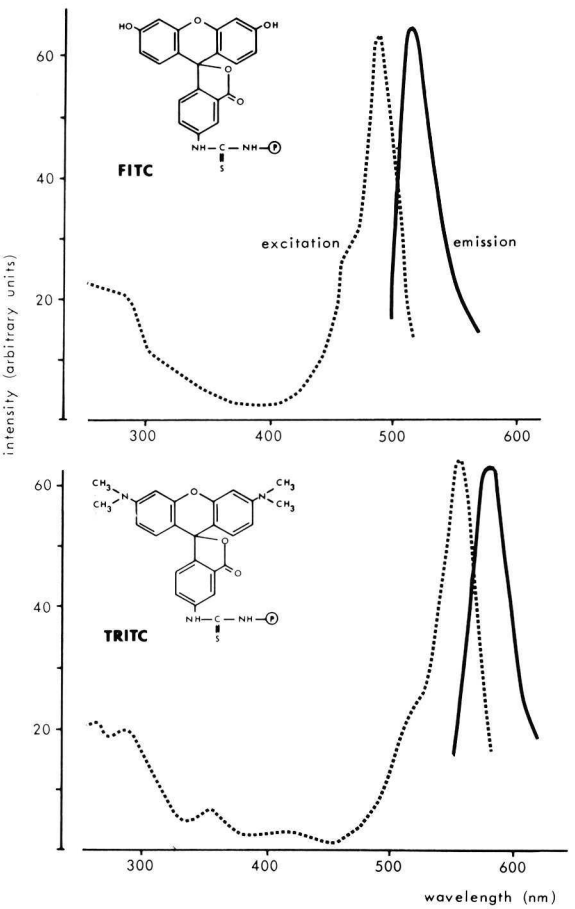


Figure 5.10. Structural formula and excitation and fluorescence spectra of fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) (reprinted with permission from Haaijman, 1977). In the formulas, P stands for protein.

and Van den Engh, 1982); and a series of new fluorochromes described by Latt and coworkers (1984).

Although the antibiotics, netropsin and distamycin, are non-fluorescent, they are used in combination with other fluorescent DNA stains to enhance chromosome banding patterns (Schweizer, 1981; Sahar and Latt, 1980) and in flow karyotyping (Meyne et al., 1984; Lalande et al., 1985). They bind nonintercalatively to the minor groove of DNA with A-T base pair specificity (Zimmer et al., 1971).

Fluorescein and rhodamine

Fluorescein and rhodamine are two fluorescent molecules that can be coupled easily to proteins. They are widely used for the visualization of bound antibody molecules to cell components. Under weakly alkaline conditions, fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) react with lysine residues in proteins via a thiocarbamide bond (Haaijman, 1977). The structures and spectra of FITC and TRITC are shown in figure 5.10. The spectra of these dyes are similar whether bound to beads or cells (Haaijman and Van Dalen, 1974).

SECTION II

ISOLATION AND STAINING OF CHROMOSOMES FOR UNIVARIATE OR BIVARIATE FLOW CYTOMETRIC ANALYSIS OF DNA CONTENT

Introduction

If meaningful and practical information is to be gained from the flow cytometric analysis of chromosomes, or if chromosomes are to be purified by flow sorting, flow karyotypes should have a high resolution and should be reproducible. Ideally, the flow karyotype shows all the chromosome types in separate peaks, and the relative peak positions are fixed. This not only depends on the precision and sensitivity of the measuring equipment. The techniques for isolating and staining chromosomes are equally important. The chromosomes should not be damaged, they should be stable once they are suspended, and they should be uniformly stained. If flow karyotyping is to be applied to the detection and study of chromosomal abnormalities, suitable chromosome suspensions must be prepared from both normal and abnormal cells.

The papers in chapters 6-10 represent the results of a series of experiments designed to decrease the variability and increase the resolution in flow karyotypes through improvements in the isolation and staining of chromosomes. In this section, chromosome identification, based on total DNA content and average base composition, is performed using single and dual laser flow cytometers. Several DNA-specific fluorochromes are employed. The chapters in this section also enlarge on the range of cell types that can be analyzed with flow karyotyping.

A procedure is described in chapter 6 that fulfills many of the requirements for an ideal chromosome isolation procedure as defined in chapter 3. A detailed study of the variables that affect a supension of chromosomes are presented. The procedure resulting from the optimalization of these variables yields chromosome suspensions for which high resolution fluorescent distributions can be measured consistently. Chromosomes isolated using this procedure can be analyzed with good results in a variety of flow cytometers.

In this procedure, Mg^{++} ions serve to stabilize the DNA and to maintain the normal morphology of chromosomes in suspension. The observation that unstained chromosomes can be stored at least 8 weeks

without loss of resolution in the measured fluorescence distribution is evidence of the stability of the isolated chromosomes. Mg^{++} ions also permit studies presented in this section of the binding of a variety of DNA-specific stains to chromatin.

In chapter 7, the isolation procedure is put to the test on human chromosomes. Analysis of human chromosomes puts high demands on the chromosome isolation procedure. Human cells contain a large number of small chromosomes, and the differences in DNA content among them are small. In chapter 7, evidence is presented to show that high quality suspensions of human chromosomes can be prepared.

The results in chapter 7 confirm earlier findings that the large differences in average base composition among human chromosomes can be used for their discrimination in a flow karyotype. If human chromosomes are stained for total DNA content and are analyzed in a single laser flow cytometer, only 15 chromosome groups can be resolved. When a dual beam flow cytometer is used to measure the two fluorescent signals from each chromosome, it is possible to identify and enumerate all the human chromosomes, except those in the 9-12 group. Distinct flow karyotype patterns, unique for different individuals, are observed. Homolog differences in several chromosomes can be quantified. Additional peaks in the flow karyotype of an in vitro colon carcinoma cell type, indicate the presence of chromosomes with abnormal base composition. This reflects the karyotypic instability of these cells.

An examination of the staining characteristics of chromatin is presented in chapter 8. These studies concern the saturation level and the kinetics of dye binding, and the interaction of DNA stains in chromatin. These observations may contribute to an understanding of the base composition of human chromosomes. The spectral interaction between the two fluorescent dyes, Hoechst 33258 and chromomycin A3, is used to assess the distribution of A-T and G-C base pairs in chromosomes.

If the unique flow karyotype patterns measured for different individuals are sufficiently reproducible, they are potentially useful in the detection of aberrant chromosomes. In chapter 8, the reproducibility of these flow karyotypes is shown to be high. The relative peak positions in the flow karyotypes measured for a given individual are approximately constant, even over long periods of time and over large variations in staining conditions.

In order to establish the usefulness of flow karyotyping for clinical studies, chromosomes must be isolated from clinical samples to consistently yield flow karyotypes with the same high resolution obtained with fibroblast cultures. It is imperative to be able to measure flow karyotypes for cells from tissues or cultures containing few mitotic cells.

Chapter 7 shows the results obtained when the cells in peripheral blood lymphocyte cultures are subjected to the chromosome isolation procedure. High quality bivariate fluorescence distributions can be measured from this cell source. The resolution obtained in the flow karyotypes is such that the distributions can be subjected to mathematical analyses. In this way, the relative frequency of different chromosomes in the cells of the donor can be determined.

Amniotic cell cultures from the clinic serve as the source of cells for chromosome isolation in chapter 9. One of the main clinical applications of karyotyping is the early diagnosis of genetic abnormalities or sex-linked hereditary disorders in the fetus. This chapter presents the results of the first successful application of flow karyotyping to clinical samples of human fetal cells. The high resolution of chromosomes in the flow karyotypes from these cells and the low level of the background continuum make it possible to use these karyotypes to determine fetal sex and to detect trisomies of chromosome 21.

Flow karyotyping also offers the capability to detect and quantify the DNA involved in the chromosomal lesions associated with leukemias. Since large numbers of chromosomes can be analyzed, it may be possible to determine the frequency of tumor cells in a cell population with this technique. For this application, however, techniques for the preparation of suitable chromosome suspensions from bone marrow cells must be developed. Bone marrow cells have a low mitotic index, even after in vivo exposure to agents that arrest cells in mitosis. It is necessary to enrich bone marrow suspensions for mitotic cells before adequate chromosome suspensions can be prepared for flow cytometric analysis. A procedure which accomplishes this is shown in chapter 10. Suspensions containing up to 63% mitotic cells can be obtained from rat bone marrow cells through density separation.

In the series of experiments presented in this section, many of the variables that have limited the use of flow cytometry in chromosome analysis in the past are defined. High resolution flow karyotypes can be obtained routinely from a variety of cell types with the techniques described here. The reproducibility of the relative peak positions in flow karyotypes of an individual confirms that enumeration and identification of chromosomes can be accomplished reliably with this technique. The DNA content of the chromosomes from clinical samples can now be analyzed. These results may eliminate many of the factors that have limited the use of flow karyotyping as a diagnostic tool in the clinic.

Chapter 6

PREPARATION OF CHROMOSOMES SUSPENSIONS FOR FLOW CYTOMETRY*

SUMMARY

Certain variables in the preparation of chromosomes suspensions for flow cytometric analysis have been investigated. The optimal conditions have been determined. The results of this series of experiments have been incorporated to yield a preparation protocol that gives chromosome profiles with a low amount of small particle debris and few chromosome clumps. The method reduces variability that results from sample preparation. Chromosomes are optimally isolated in a hypotonic solution buffered to pH 8.0. $MgSO_4$ and dithiothreitol added to the buffer reduce the number of clumps and small fluorescent particles. The presence of $MgSO_4$ also stabilizes the chromosomes and precludes the need for other stabilizing agents such as propidium iodide. When the swelling buffer developed in this investigation is used, unstained chromosomes are stable for at least 1 wk if stored at 4°C. The preparation procedure can be used with the DNA stains, propidium iodide, Hoechst 33258, and mithramycin. Preliminary experiments show that this procedure can also be used for bivariate analysis of human and Chinese hamster chromosomes. The importance of this improvement for studies on chromosome damage caused by irradiation or mutagens is discussed.

INTRODUCTION

The ability to analyze rapidly and quantitatively and to sort mammalian chromosomes with flow cytometry has generated considerable interest from cytogeneticists, molecular biologists and radiobiologists. Several methods have been described for the preparation of chromosome suspensions (Bijman, 1983; Matsson and Rydberg, 1980; Otto et al., 1980; Sillar and Young, 1981; Stöhr et al., 1982; Wray and Stubblefield, 1970; Yu et al., 1981). Chromosome suspensions are generally prepared by: 1) blocking and collecting cells in mitosis, 2) swelling hypotonically,

*This chapter is adapted from a paper published by Ger van den Engh, Barb Trask, Scott Cram and Marty Bartholdi in Cytometry 5:108-117 (1984).

3) disrupting mechanically in the presence of a detergent, and 4) staining with a fluorescent DNA-specific dye. Not all procedures are designed or are expected to work equally well on all types of cells.

At the Los Alamos laboratory, the method described by Aten et al. (1980) has been found to yield good results (Bartholdi et al., 1983). In this method, the swelling medium is 75 mM KCl to which propidium iodide (PI) has been added. The addition of PI at the swelling step has been assumed to help stabilize the chromosome structure. The cells are ruptured by syringing in the presence of a neutral detergent (Triton X-100). Subsequently, the chromosomes and cell fragments are treated with RNase.

In general, this method yields good to excellent chromosome preparations. There is a degree of unpredictable day-to-day variation, which makes the interpretation of small changes in flow karyotypes difficult. Evidently, some of the variables that play a role in chromosome quality are not controlled stringently enough.

Ideally, a histogram of the DNA content of chromosomes (a flow karyotype) consists only of a number of peaks. The peak position indicates the DNA content of the chromosome. The width of the peak is determined by the uniformity of chromosome staining, the uniformity of DNA content per chromosome, and the precision of the flow cytometer. Actual flow karyotypes contain broad distributions underlying the chromosome peaks. This background is caused by clumps of two or more chromosomes, chromosome fragments, and other unidentified fluorescent particles.

The experiments described here were designed to determine whether changing the conditions during chromosome preparation would minimize these background signals. A number of factors were identified that influence chromosome preparation quality, and the procedure was changed to optimize these parameters. The resulting protocol (table 6.1) consistently yields chromosome preparations of good quality and can be used for the staining of chromosomes with a variety of DNA fluorophores.

MATERIALS AND METHODS

Cell cultures

A tissue culture cell line initiated from Chinese hamster fetuses between 14 and 16 days of gestation (WCHE/5 clone 2, a tetraploid line at approximately passage number 60) was used in all experiments. The cells were maintained in exponential growth in alpha-MEM medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum in 75-cm^2 culture flasks.

General procedure

The general procedure used to prepare chromosomes for flow cytometry was as follows. The contents of the hypotonic solution and other variables in the procedure were varied systematically. The effects of these variations are presented in Results.

Growth medium in culture flasks was replaced by 10 ml alpha-MEM medium containing the mitotic arresting agent (colcemid or vinblastine). Cells were blocked for 4 h. Mitotic cells were collected from several flasks by tapping against the side of the flasks. The cells were combined and redistributed in test tubes. The suspensions were centrifuged at 150g for 8 min. One ml of the hypotonic swelling medium was added to the cell pellet, and the tube was lightly agitated. After 10 min at room temperature, 0.1 ml Triton X-100 (0.25-0.30% final concentration) was added. After 10 min at room temperature, the suspension was syringed forcefully through a 22- or 25-gauge needle. This was followed by 30 min of incubation at 37°C for RNase action. The swelling media were freshly made at most 1 d in advance and were filtered through 0.22-μm filters before use. Stock solutions of propidium iodide (PI) and RNase were made at 20 times final concentration in distilled water and 75 mM KCl, respectively. PI and RNase were added to the hypotonic swelling medium before filtration.

Table 6.1

PROCEDURE FOR PREPARATION OF CHROMOSOMES

1. Block cells in mitosis with vinblastine (5×10^{-7} M) or colcemid (3×10^{-7} M) for 3 to 4 h.

2. Harvest mitotic cells by shaking off, and centrifuge at 150g for 8 min. Remove all supernatant fluid.

3. Add 1 ml swelling buffer to pellet and let stand 10 min at room temperature. Swelling buffer: 50 mM KCl, 10 mM $MgSO_4$, 20 μg/ml PI, 0.25 mg/ml RNase, 3 mM dithiothreitol, and 5 mM K_2HPO_4 or 5 mM HEPES adjusted to pH 8.0 (addition of the DNA stain can be delayed until after preparation with the same results).

4. Add 0.1 ml Triton X-100 to a final concentration of 0.25%, and leave for 10 min at room temperature.

5. Forcefully syringe three times (22 or 25 gauge needle) and incubate at 37°C for 30 min.

6. Measure, or store at 4°C (after addition of NaN_3, 3 mM final concentration).

PI staining of the chromosomes was measured on a flow microfluorometer (Los Alamos National Laboratory's FMF II). Laser power was 800 mW tuned to 488 nm (Laser model 164, Spectra Physics, Mountain View, CA). The photomultiplier voltage was 800 V in all cases. The amplifier gain was adjusted for each run to utilize the 256 channels of the analyzer. In the FMF II, the size of the illuminating light spot is in the same order of magnitude as the length of the largest chromosomes. Consequently, the height of the pulses from the photomultiplier is not solely dependent on the DNA content of the chromosomes, but is also influenced by chromosome size. In order to obtain signals that are proportional to chromosomal DNA content, the pulses were integrated over the duration of the pulse using a self-gated integrating amplifier (model 8124) built at Los Alamos. The distributions shown in figure 7 were measured on the high-resolution flow cytometer at Los Alamos (for details, see Bartholdi et al., 1983).

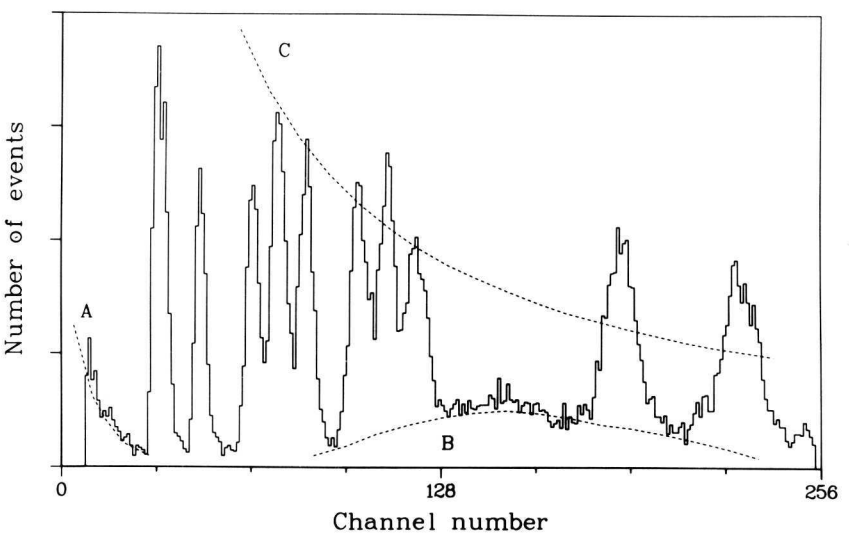


Figure 6.1. Fluorescence distribution of a chromosome suspension of a Chinese hamster cell line. The chromosomes were prepared using a modification of the method described by Aten et al. (1980). This particular profile was chosen since it illustrates the quality criteria for a flow karyotype. Usually, profiles of a much better quality are obtained by the Aten procedure. The cell pellet was treated with 1 ml of a solution containing 75 mM KCl and 50 µg/ml PI. After 10 min, 0.5 ml of a solution containing 75 mM KCl, 50 µg/ml PI, 1 mg/ml RNase and 1% Triton X-100 was added. This was followed by syringing and incubation at 37°C. The peaks represent the 11 pairs of Chinese hamster chromosomes. The peaks represent (from right to left) chromosomes numbers 1, 2, 3, X and 4 through 10, respectively. Chromosome 9 and 10 are combined in the first peak on the left. The coefficient of variation for chromosomes 1 and 2 is approximately 3%. The dashed line at A represents small particle noise. If all the chromosome peaks contain an equal number of events and have the same coefficient of variation, their height should follow a line as given by C.

Chemicals used

Mitotic arrest agents: vinblastine (Velban, Eli Lilly and Co., Indianapolis, IN) and colcemid (GIBCO, Grand Island, NY). Ribonuclease: bovine pancreatic (2724 units/mg, Worthington Biochem. Corp., Freehold, NJ). Buffers: Hepes, Tris (both GIBCO), and phosphate (K_2HPO_4). Detergents: Triton X-100, Saponin (both Sigma Co., St. Louis, MO) and digitonin (Calbiochem, La Jolla, CA). DNA stains: propidium iodide, Hoechst 33258 (both Calbiochem), mithramycin (Pfizer Diagnostics Div., NY), and netropsin (Eli Lilly). Other agents: β-mercaptoethanol (Eastman Kodak Co., Rochester, NY), spermine, dithiothreitol, and p-chloromercuriphenylsulfonic acid (all Sigma). All other chemicals used were analytical grade.

RESULTS

Certain aspects in the chromosome preparation procedure were varied to investigate their importance in obtaining good flow karyotypes. The conditions that gave the best results are summarized in table 6.1. The experiments that led to this procedure are described in detail in this section. The results presented are representative of 2-4 repeated experiments.

Criteria for the quality of a chromosome preparation

In the experiments described here, conditions were sought that consistently yielded flow karyotypes with 1) peaks with low coefficients of variation, 2) low small-particle debris, 3) few chromosome clumps, and 4) representation of chromosomes proportional to their numbers in cells. These criteria are illustrated in figure 6.1. This flow karyotype was chosen for illustrative purposes only; the modified Aten procedure used in its preparation (see legend) routinely yields better distributions (see Bartholdi et al., 1983).

Chromosomes in good preparations should be uniformly stained giving peaks with low coefficients of variation (CV). A good preparation should contain relatively little small-particle debris and few clumps. These particles affect the chromosome distribution in two areas. From channel 1 onward, there is a rapidly decreasing contribution of small particles (line A in figure 6.1). Clumps of chromosomes result in a broad band in the higher channels (line B). The chromosome clumps peak between chromosomes 2 and 3. Experience has shown that these chromosome clumps represent a failure to disperse completely the cell fragments rather than the aggregation of chromosomes after syringing. If chromosomes are present in equal numbers in the cells, and if the coefficient of variation of all the

peaks is the same, and if the number of background counts is low, peak height should be inversely proportional to channel number (and should follow line C). In the preparation shown in figure 6.1, the smaller chromosomes are underrepresented compared to the numbers of larger chromosomes. This is often the case with preparations that contain a relatively large number of clumps. The clumps tend to contain more small chromosomes than large ones. The larger chromosomes apparently are more readily dispersed. A good preparation procedure should not select for certain chromosome types. Rather, the relative abundance of the various chromosomes should accurately reflect the chromosome numbers present in the cells.

Most of the mathematical procedures used to analyze flow karyotypes are based on the estimation of the coefficient of variation of the individual chromosome peaks. There are no generally accepted procedures

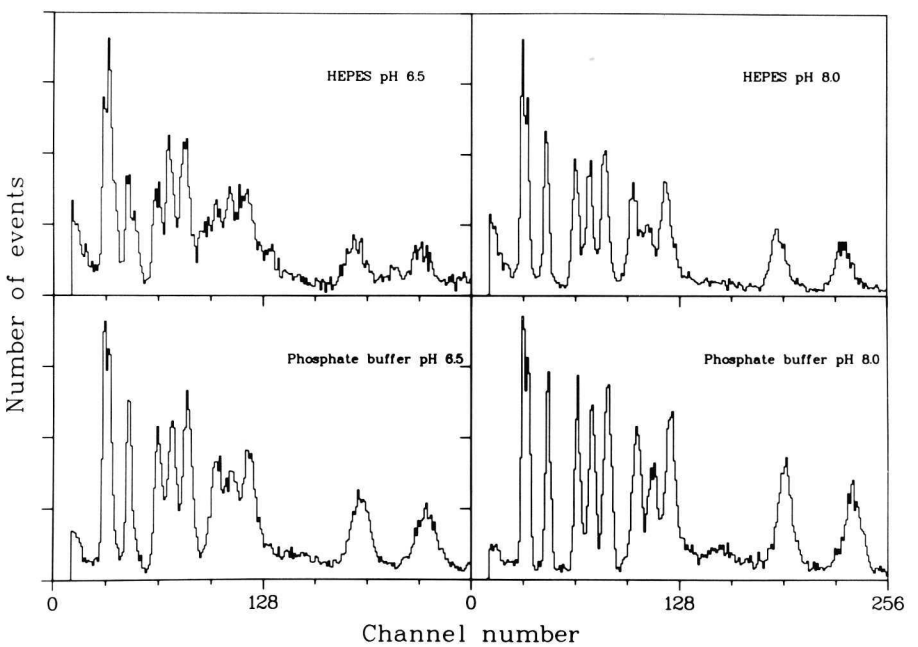


Figure 6.2. Effect of the pH of the swelling buffer on chromosome distributions. The panels on the left represent fluorescence distributions of Chinese hamster chromosomes prepared at pH 6.5. The suspensions shown on the right were prepared at pH 8.0. In the top panels, 5 mM HEPES was used as the buffer; in the bottom panels, 5 mM K_2HPO_4. The hypotonic buffer for these experiments contained 5 mM buffer, 75 mM KCl, 50 µg/ml PI, and 0.25 mg/ml RNase. Triton X-100 concentration was 0.25%.

for determining the amount of small-particle debris or the number of chromosome clumps. Since the latter aspects are equally important in judging the quality of a chromosome preparation, a subjective evaluation of the the flow karyotypes based on the previously described criteria was used instead of a mathematical approach.

Effect of pH

The pH of the sample was suspected to vary greatly in the standard procedure. Variation in the amounts of supernatant left after centrifugation and variation in the time of exposure of the carbonate-growth medium to air during preparation could yield differences in pH from sample to sample. The standard swelling medium of 75 mM KCl was supplemented with a pH buffer to investigate whether the pH at the time of swelling affects chromosome quality. PI (50 μg/ml final concentration) and RNase (0.25 mg/ml final concentration) were added to the swelling buffer. Three buffers were tested: phosphate, Hepes, and Tris. In each case the buffer concentration was 5 mM. The pH was varied in steps of 0.5 from 6.5 to 8.5. Preparations made at pH 8.0 were qualitatively the best, while chromosome profiles made at pH 6.5 showed many clumps and were characterized by broad peaks (figure 6.2). At pH 8.0, few clumps are encountered. Microscopic examination of the preparations indicated that the clumps at low pH were due to incomplete disruption of the cells. The same variation in chromosome preparation quality with a change in pH was found with each of the three buffers. Hepes and phosphate buffers gave better chromosome distributions than did the Tris buffer. In all further experiments, the hypotonic swelling medium was buffered to pH 8.0. Because of the chance of precipitate formation in the phosphate-buffered hypotonic solution, Hepes buffer is recommended when storage of the solution or the prepared chromosomes is planned.

RNase: concentration and time of addition

The effect of RNase was studied by adding the enzyme to a swelling medium consisting of 75 mM KCl, 50 μg/ml PI, and 5 mM Hepes at pH 8.0. The addition of RNase considerably reduces the amount of small fluorescent particle debris (figure 6.3), which can be attributed to PI staining of double-stranded RNA. Best results were obtained if the RNase was present during the swelling of the cells. If the RNase was added with the Triton X-100 or after syringing, more small-particle debris was found.

The concentration of RNase (Worthington, 2724 units/mg) was varied from 0.01 to 1 mg/ml. At the highest concentration, some clumping was observed. Optimal results were obtained at concentrations of 0.1 and 0.3 mg/ml.

It is common practice to boil RNase before use in order to remove any contaminating DNase. At least with the RNase preparation used here, this was found to be unnecessary. In fact, boiling reduced RNase activity and added background signals to chromosome preparations. In all experiments reported here, unboiled RNase was used.

Detergents: type and timing

The effectiveness of three detergents (digitonin, saponin, and Triton X-100) in dissolving the cell membranes and in aiding the disruption of the

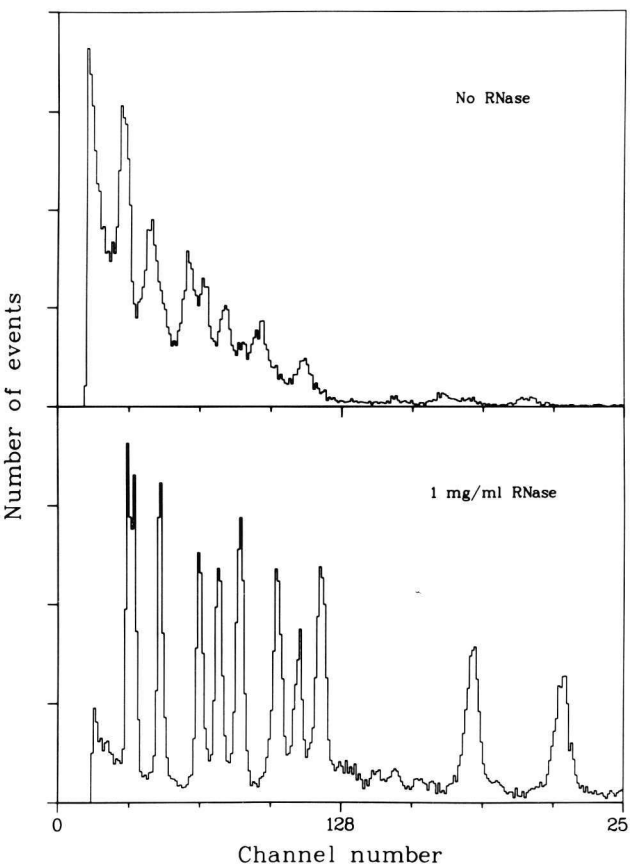


Figure 6.3. Effect of RNase on chromosome suspensions. The fluorescence profile of a chromosome suspension without RNase treatment is dominated by small particle fluorescence (top panel). The bottom panel shows the improvement in peak resolution after 30 min RNase treatment at 37°C (1 mg/ml). The hypotonic buffer for both panels contained 75 mM KCl, 5 mM HEPES, and 50 µg/ml PI. Triton X-100 concentration was 0.3%.

cell contents was investigated. After the cells had been in the swelling buffer for 10 min at room temperature, 0.1 ml of 10x concentrated detergent was added. Saponin (Sigma) appeared to contain a fluorescent contaminant. Although this did not influence the results at low concentrations, saponin was not used for further experiments for this reason. Both digitonin and Triton X-100 gave good results. Triton X-100 is perhaps the better choice of the two detergents for two reasons: 1) its chemical composition is better defined than that of digitonin, and 2) it spares mitochondrial membranes, whereas digitonin, a cationic detergent, releases the mitochondrial contents into the suspension. The final Triton X-100 concentration was varied from 0.01% to 1%. Concentrations of 0.1% and 0.3% gave the best results.

Addition of inorganic salts

Inorganic salts were added to the swelling buffer to investigate their effect on the quality of the chromosome preparations. Those ions that are the main constituents of intracellular fluid were of particular interest. Because Mg^{++} ions are necessary when staining DNA with the chromomycin family of dyes, their addition to the swelling buffer was thought to be desirable. The salts tested ($CaCl_2$, $MgSO_4$, and $MgCl_2$) were added at concentration of 10 mM (5 mM for $CaCl_2$) to a solution of 75 mM KCl, 5 mM Hepes, 50 µg/ml PI, and 0.25 mg/ml RNase at pH 8.0. $CaCl_2$ and $MgCl_2$ were found to have no effect. The addition of 10 mM $MgSO_4$ consistently reduced the number of clumps in the chromosome preparation. This was the case when the hypotonic buffer contained either 50 mM or 75 mM KCl and either 5 mM K_2HPO_4 or 5 mM Hepes as buffer. The presence of $MgSO_4$ markedly improved the chromosome preparations made at both pH 6.5 and 8.0.

Addition of reducing agents

The addition of beta-mercaptoethanol or dithiothreitol to either Hepes- or K_2HPO_4-buffered swelling medium was found to reduce the number of clumps in the preparations (figure 6.4). If $MgSO_4$ was present, this effect was less pronounced, since addition of $MgSO_4$ alone reduces the number of clumps. Good results were obtained at concentrations of 3 mM β-mercaptoethanol and 1-3 mM dithiothreitol. The two chemicals were prepared as 30 times concentrated stock solutions in water and were added to the swelling buffer shortly before use. Since either compound could conceivably interfere with RNase activity, the importance of the time of addition was investigated. Addition with the detergent or after syringing gave less satisfactory results than addition with the swelling buffer.

Other additives

Spermine has been reported in the literature to have a stabilizing effect on chromosomes (Sillar and Young, 1981). The addition of spermine was tested to determine its effect in Hepes-buffered swelling medium (5 mM Hepes, 75 mM KCL, 1 mg/ml RNase, 50 µg/ml PI at pH 8.0). Spermine was found to decrease resolution under the conditions of this procedure.

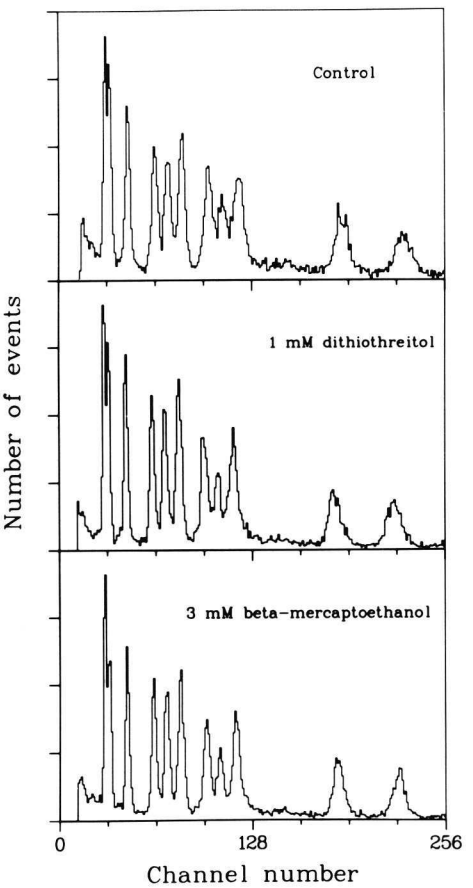


Figure 6.4. The effect of the addition of dithiothreitol and beta-mercaptoethanol on the fluorescence distribution of Chinese hamster chromosomes. The top panel shows chromosomes prepared with 75 mM KCl, 5 mM HEPES, 50 µg/ml PI, and 1 mg/ml RNase at pH 8.0. Triton X-100 concentration was 0.25%. The middle and bottom panels show the distributions obtained with the addition of 1 mM dithiothreitol and 3 mM beta-mercaptoethanol, respectively. As is the case when $MgSO_4$ is added, a reduction in clumps is observed. This is most apparent in the deepened valley between chromosomes 2 and 3 and in the improved resolution of chromosomes 3, X, and 4.

Concentrations of 0.8, 2.8, and 8 mM were tested. As the spermine concentration was increased to 8 mM, the results became progressibly worse.

Chloromercuriphenylsulphonic acid is a compound that oxidizes sulfhydryl-bonds and dissociates fibrin structures (Kuriyama and Sakai, 1974). Its addition to the swelling buffer at concentrations of 1, 3, and 10 mM was found to have a negative influence on the quality of chromosome preparations.

Cell concentration

Chromosome preparations were made using different numbers of cells (ranging from approximately $2x10^4$ to 10^6 cells). The number of cells/ml hypotonic buffer is not an important factor in the resolution for the flow karyotype.

Vinblastine and colcemid for the induction of mitotic arrest

Vinblastine and colcemid were found to be effective agents for accumulating cells in mitosis. Vinblastine concentrations of 0.05 μM, 0.17 μM, and 0.5 μM were tested. Colcemid was tested at 0.03 μM, 0.09 μM, and 0.3 μM. The optimal concentrations were 0.5 μM and 0.3 μM vinblastine and colcemid, respectively. At the optimal concentrations, there was no difference between the two spindle poisons in the quality of the chromosome preparations. Inclusion of colcemid in the swelling buffer (0.1 μg/ml) did not significantly improve the results.

Ionic strength of the swelling buffer

The ionic strength of the swelling buffer was varied by adding various concentrations of KCl to 5 mM Hepes, 50 μg/ml PI, and 1 mg/ml RNase at pH 8.0. The KCl concentration was varied from 25 to 150 mM. Low KCl concentrations caused total destruction of the chromosomes. High KCl concentrations caused incomplete disruption of the cells. The optimal KCl concentration was found to be between 50 and 75 mM.

The effect of osmolarity was also studied with a $MgSO_4$- and dithiothreitol-containing medium. A buffer consisting of 5 mM Hepes, 50 mM KCl, and 10 mM $MgSO_4$ (at pH 8.0) was diluted with varying amounts of 5 mM Hepes (pH 8.0). The resulting KCl concentrations were 10, 20, 30, 40, and 50 mM. The different groups received the same concentrations of dithiothreitol, RNase, and propidium iodide (3 mM, 0.25 mg/ml, and 20 μg/ml final concentrations, respectively). Chromosomes prepared at these different osmolarities were all low in small-particle debris and chromosome clumps. However, the chromosome peaks were significantly broader at the lower osmolarities. Again, 50 mM KCl and 10 mM $MgSO_4$ were concluded to be the optimal salt concentrations.

Propidium iodide: concentration and timing

The concentration of PI in the swelling buffer was varied from 0.5 to 150 µg/ml. In this series of experiments, the swelling buffer contained 50 mM KCl, 5 mM Hepes, and 0.25 mg/ml RNase at pH 8.0. The relative fluorescence of the chromosomes did not increase at concentrations above 20 µg/ml, but background signals due to small fluorescent debris increased above 50 µg/ml. The optimal PI concentration was determined to be 20 µg/ml.

It was investigated whether PI is necessary to stabilize the chromosomes during preparation by varying the interval between cell disruption and the addition of PI. In medium containing $MgSO_4$ and dithiothreitol (at pH 8.0), the presence of PI is not essential to obtain

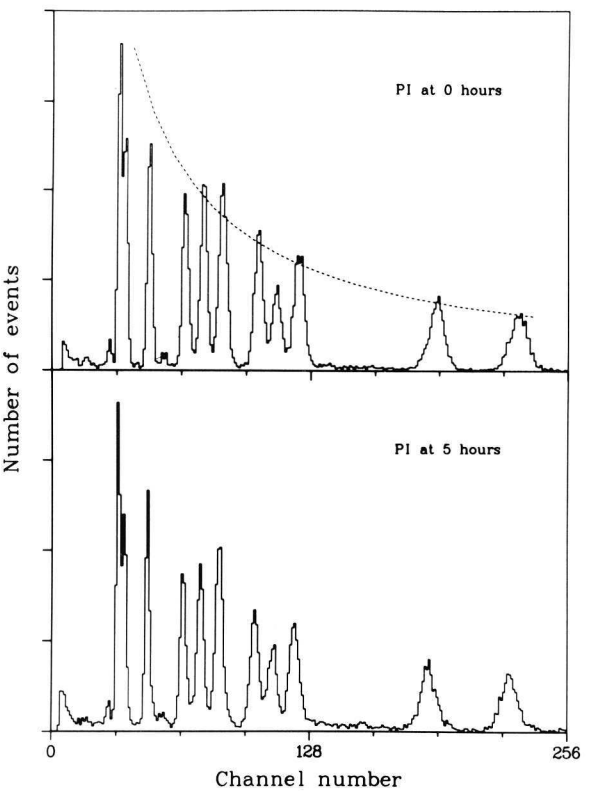


Figure 6.5. A flow karyotype of Chinese hamster chromosomes prepared according to the procedure described in table 6.1. In the chromosome preparation depicted in the top panel, PI was added to the cell pellet with the swelling buffer (at 0 h). The chromosomes shown in the bottom panel were prepared using the same procedure, but in the absence of a DNA stain. The unstained chromosomes were kept at 4°C for 5 h before PI was added.

good flow karyotypes. Figure 6.5 shows a flow karyotype obtained using medium in which PI was present at the start of the procedure (top panel). The bottom panel shows the flow karyotype obtained when the addition of PI was delayed until 5 h.

Storage

Unstained or stained chromosomes prepared according to the protocol in table 6.1 have been kept up to 8 wk at 4°C without appreciable degradation of chromosome quality. NaN_3 was added (3 mM final concentration) to stained chromosomes to prevent bacterial growth. During storage, chromosomes settle to the bottom of the container. Gentle syringing separates the sedimented chromosomes before measurement.

When MgSO4 is omitted from the preparation buffer, another agent (such as PI) is necessary to stabilize chromosomes for storage. In the absence of both PI and $MgSO_4$, chromosome quality deteriorates rapidly.

Other DNA stains

The procedure described in this paper (table 6.1) has also be used with other DNA stains. The distributions obtained using the high illumination flow cytometer at Los Alamos for PI-, mithramycin-, and Hoechst 33258-stained chromosomes are shown in figure 6.6. Propidium iodide, mithramycin, and Hoechst 33258 concentrations were 20 μg/ml, 12.5 μg/ml, and 5 μg/ml, respectively. In this case, the DNA stains were added directly to the hypotonic buffer; staining can also be delayed until after preparation with the same results.

DISCUSSION

Some of the factors that influence the quality of chromosome preparations were identified in the foregoing experiments. The conditions we found to be optimal were incorporated in the protocol described in table 6.1. The top panel in figure 6.5 shows a chromosome preparation of Chinese hamster tissue culture cells that have been prepared according to this procedure. Note the low level of small-particle noise and the absence of clumps in the gap between chromosomes 2 and 3.

If no preferential loss of certain chromosomes has occurred during preparation, the peak height of each chromosome should be inversely proportional to the channel number (assuming two copies of each chromosome in each cell). Using this preparation protocol, there is a fair representation of the various chromosome types. The peaks heights are approximately inversely related to the peak position (and follow the dashed line). The cells depicted here contain only one X chromosome, which is

reflected in the low height of the fourth peak from the right. Due to limitations of the measuring equipment, the coefficients of variation of the peaks of the smaller chromosomes are larger than those of the large chromosomes. This may account for the fact that the first three peaks are somewhat low in comparison to the others.

In comparing figure 6.5 with figure 6.1, it will be clear that the coefficient of variation of the chromosome peaks is inadequate when used alone as a criterion for chromosome quality. The coefficient of variation of chromosome 2 in figure 6.1 as judged from the halfwidth at 50% peak height is approximately 3%. The coefficient of variation of chromosome 2 in figure 6.5 is approximately 2%. Yet the preparation in figure 6.5 is clearly better when comparisons are based on other criteria.

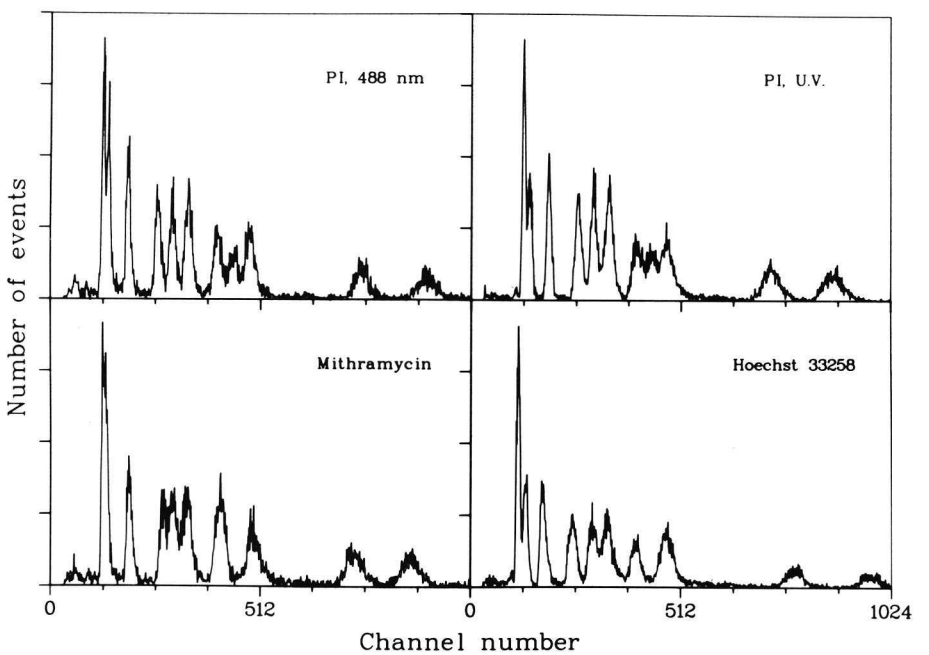


Figure 6.6. Flow karyotypes of Chinese hamster chromosomes using various DNA stains. The chromosomes were prepared according to the procedure in table 6.1. The histograms in the right two panels were made on a different day (and therefore at a different passage) than the two panels on the left. In the top two panels, PI (20 µg/ml) was used, excitation wavelengths were 488 nm and UV (351 and 364 nm), for the left and right panels, respectively. In the bottom left panel, mithramycin (12.5 µg/ml) was used, excitation wavelength was 458 nm. In the bottom right panel, Hoechst 33258 (5 µg/ml) was used, excitation in the UV.

The pH of the swelling medium is an important factor in the preparation of chromosome suspensions. Preparations prepared at pH 7.0 or lower showed broader peaks and contained more clumps and small fluorescent particles than those prepared at pH 8.0. The importance of pH may explain some of the variability that has been encountered when unbuffered swelling media are used. Tissue culture cells are grown in media containing a carbonate buffer. When exposed to air, these media can change in pH from 7 to 8. The speed at which the cells are processed and the relative volume of the medium carried over with the cell pellet into the swelling buffer may affect the pH during swelling. The presence of a pH buffer in the swelling medium eliminates this variability and improves the reproducibility of the method. Both Hepes and K_2HPO_4 buffers were found to perform satisfactorily.

The presence of $MgSO_4$ and/or a reducing agent, such as dithiothreitol, greatly reduces the number of clumps in a preparation. These substances also improve chromosome suspensions prepared at low pH. Microscopic examination of the suspensions has led to the conclusion that high pH, $MgSO_4$, and reducing conditions decrease the forces necessary to shear and disrupt the cells, thereby reducing the chance of chromosome breakage.

Recently, Bijman (1983) reported success with buffers of low ionic strength. In our experience, osmolarities lower than 50 mM lead to a broadening of the chromosome peaks. In our case, reduction of background signals was achieved by the addition of $MgSO_4$, RNase, and a reducing agent. The effect of RNase can be be expected to be less pronounced when stains with a low affinity for double-stranded RNA are used.

The results obtained using other DNA stains and the observation that unstained chromosomes can be stored for at least 8 wk with no appreciable loss in chromosome quality indicate that $MgSO_4$ can replace PI as a stabilizing agent.

Flow cytometric analysis of chromosomes is being used as a measure of chromosome damage caused by radiation or chemical mutagens. The shape of the flow karyotypic profile after various doses of exposure serves as a measure of chromosome damage. A protocol such as the one described in table 6.1 is essential to determine accurately the baseline levels of clumps and small-particle debris. The number of clumps and small fluorescent particles present in a chromosome suspension is affected by the swelling conditions. This observation may have serious implications. In assays for chromosomal damage, different samples contain different numbers of dead cells, cell debris, and dividing cells. At this stage, it is difficult to predict how this will affect the chromosomes in suspension. If, for instance, the number of clumps increases with the number of dead

cells, the observed changes in chromosome profiles are only indirectly related to the agent being studied and are not due to direct chromosome damage. The chromosome clumps are found in specific regions of the chromosome distribution. It is in these areas where special care must be taken before inferring damage due to mutagens or other agents.

The procedure developed as a result of the experiments described in this paper optimizes variables important for chromosome preparation. Use of this procedure eliminates much of the variability in results (chapter 8). In addition, chromosomes can be stored after addition of NaN_3 for at least 8 wk at 4°C with good results. The procedure has also been used with DNA stains other than PI. Although the isolation procedure was developed with Chinese hamster cells, it has also been used successfully on several cell lines of human origin (chapter 7). Preliminary studies show that the method can also be used to study the competition between netropsin, a non- fluorescent DNA intercalator, and other fluorescent DNA stains. Good chromosome peak distributions have been obtained using Hoechst 33258 and mithramycin. These dyes can also be used in combination for bivariate analysis.

Chapter 7

PREPARATION OF CHROMOSOME SUSPENSIONS FOR FLOW CYTOMETRY
II. BIVARIATE ANALYSIS OF HUMAN CHROMOSOMES*

SUMMARY

Chromosomes were isolated from a variety of human cell types using a HEPES-buffered hypotonic solution (pH 8.0) containing KCl, $MgSO_4$, dithioerythritol and RNase. The chromosomes isolated by this procedure could be stained with a variety of fluorescent stains including propidium iodide, chromomycin A3 and Hoechst 33258. Addition of sodium citrate to the stained chromosomes was found to improve the total fluorescence resolution. High quality bivariate Hoechst vs. chromomycin fluorescence distributions were obtained for chromosomes isolated from a human fibroblast cell strain, a human colon carcinoma cell line and human peripheral blood lymphocyte cultures. The Hoechst vs. chromomycin flow karyotypes of a given cell line, made at different times and at dye concentrations varying over fourfold ranges, show little variation in the relative peak positions of the chromosomes. The quality of the chromosome suspensions is such that the detail that is observed in the flow karyotype is limited by the resolution of the flow cytometer and the mode of data acquisition. The size of the DNA extracted from chromosomes isolated using this procedure ranges from 20 to over 50 kilobase pairs. The described isolation procedure is simple, it yields high quality flow karyotypes, and it can be used to prepare chromosomes from clinical samples.

INTRODUCTION

Several methods for the preparation of chromosome suspensions for flow cytometry have been described (Aten et al., 1980; Blumenthal et al., 1979; Collard et al., 1984; Matsson and Rydberg, 1980; Sillar and Young, 1981; chapter 6; Wray and Stubblefield, 1970). All these methods have been shown to yield good flow karyotypes. However, they do differ in other aspects.

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*This chapter is adapted from the paper published by Ger van den Engh, Barb Trask, Joe Gray, Rich Langlois, and Loh-Chung Yu in Cytometry (1985) 6:92-100.

There are differences in the ease of preparation, the amount of small particle debris, the number of cells required, and the resultant chromosome size. For example, the polyamine method (Blumenthal et al., 1979; Sillar and Young, 1981) yields stable chromosomes that have been successfully used to create chromosome specific DNA libraries (Davies et al., 1981; Lalande et al., 1984). The chromosomes that are obtained with this method are very compact and are not easily banded (Buys et al., 1982). Other methods yield elongated chromosomes that can be easily identified under the microscope (Yu et al., 1981). Most methods seem to work best with large numbers of cells. Depending on the application for which the chromosomes are prepared, some methods will be more suitable than others.

This paper presents measurements of chromosomes prepared with an isolation procedure in which $MgSO_4$ is used to stabilize the DNA of the chromosomes (chapter 6). This method was developed for the preparation of chromosomes from Chinese hamster cells and has several desirable features: (1) The chromosomes can be stained with a variety of DNA stains, (2) the preparations have a low background due to small fluorescent particles, and (3) unstained chromosomes can be stored in the isolation buffer for several weeks without appreciable reduction in the quality of the chromosome fluorescence distributions. We now show that the procedure is also useful for the isolation of chromosomes from human cells for bivariate flow karyotype analysis using the DNA stains, Hoechst 33258 (HO) and chromomycin A3 (CA3) (Gray et al., 1979b; Langlois et al., 1982). This application puts high demands on the isolation procedure. Human chromosomes are smaller than Chinese hamster chromosomes, and differences in DNA content among them are small. Consistently low peak coefficients of variation and low background levels are required to resolve the human chromosomes. For the application of flow karyotyping as a diagnostic tool, it is also essential that good preparations can be obtained from cultures containing few mitotic cells or with a low mitotic index. In order to establish its usefulness for clinical applications, the isolation procedure is applied to cultures of peripheral blood lymphocytes. We also show that the size of the DNA fragments extracted from the isolated, stained chromosomes is sufficiently large for most recombinant DNA cloning procedures.

MATERIALS AND METHODS

Cell lines, cell culture and mitotic cell collection

Human chromosomes were prepared from 1) a human diploid fibroblast culture, LLL (Lawrence Livermore Laboratory) strain 811, derived from foreskin; 2) a cell culture derived from a human colon carcinoma (LOVO, a

gift from Dr. B. Drewinko, M.D. Anderson Tumor Institute); and 3) phytohemagglutinin-stimulated human peripheral lymphocytes. The first two human cell types were maintained in exponential growth in 75- or 150-cm^2 culture flasks containing alpha-minimum essential medium (MEM) (Gibco, Grand Island, NY) supplemented with 15% fetal calf serum (FCS, Gibco). The method for culturing peripheral lymphocytes is described by Yu et al. (1981). Briefly, lymphocytes were separated from 12 ml peripheral blood with lymphocyte separation medium (Litton Bionetics, Kensington MD), stimulated in phytohemagglutinin-M (PHA, Gibco) and cultured in small glass flasks for 72 h at 37°C.

The number of mitotic cells collected for chromosome isolation was increased in all cultures by the addition of colcemid for extended periods. LLL 811 and LOVO cells were blocked in mitosis when the cultures were at a cell density of approximately 50% confluency. Growth medium was removed and alpha-MEM medium supplemented with FCS and containing 0.1 μg/ml colcemid (Gibco) was added (10 ml per 75 cm^2 flask). Lymphocytes were blocked after 72 h incubation by the addition of colcemid directly to the cultures (final concentration 0.1 μg/ml). Cells were incubated at 37°C in the presence of colcemid for 10-12 h. Mitotic cells of LLL 811 and LOVO cell cultures were harvested by shake-off. This enrichment of mitotic cells was not possible with peripheral lymphocyte cultures; the entire contents of the culture vials were collected.

Chromosome isolation

Aliquots of cells (approximately 10 ml for LLL 811 or LOVO, containing about 10^5 mitotic cells) or 7 ml for peripheral lymphocyte cultures (contents of 1 culture vial) were centrifuged and resuspended in isolation buffer as described in chapter 6 for the isolation of chromosomes from Chinese hamster cells. After isolation, the chromosomes were stained with 2.7 μM Hoechst 33258 (HO) and 26 μM chromomycin A3 (CA) (both from Sigma). The chromosome suspensions were stored on ice after staining. Except where noted, sodium citrate (Na-citrate) was added to the chromosomes 2 h after staining to a final concentration of 10 mM. Analysis of the chromosomes was performed at least 2 h after staining to allow time for the dyes to equilibrate.

Molecular weight determination

The molecular weight of the DNA isolated from chromosome suspensions before and after cell sorting was determined by gel electrophoresis (Yu et al,. 1981). The sorted chromosomes were frozen immediately after sorting and stored at -70°C until analysis. The unsorted chromosomes were analyzed less than 24 h after isolation. The chromosomes were concentrated by

centrifugation, resuspended in DNA isolation buffer (150 mM NaCl; 10 mM Tris-HCl, pH 8.0; and 10 mM EDTA) containing 0.5% SDS and 0.5 mg/ml proteinase K and incubated overnight at 37°C. The DNA was then extracted, precipitated in cold ethanol, and resuspended in 10 mM Tris plus 1 mM EDTA.

The DNA samples isolated from sorted and unsorted chromosomes and from HindIII digested lambda phage (fragments of 23.5, 9.7, 6.6, 4.3, 2.2 and 2.1 kbasepairs (kb) were then analyzed in separate lanes on a 0.4% agarose gel made up in Tris-acetate buffer with 0.5% ethidium bromide.

Flow cytometric analysis

Flow cytometric analysis of the chromosome suspensions was performed on the LLL dual beam flow cytometer equipped with two lasers (model 164 for visible light, model 171 for UV light, Spectra Physics, Mountain View, CA). Chromosomes were forced by a sheath fluid of distilled water to flow one by one through two spatially separated laser beams. One laser was adjusted to emit at 458 nm (200 mW laser power) to excite CA3, and the resulting fluorescence was measured through a 480 nm long pass filter (3-71, Corning). The second laser was adjusted to emit in the ultraviolet range (351 and 364 nm) with 1 W laser power (except where noted otherwise) to excite HO. HO fluorescence was measured through a 425 nm high pass filter (Ditric Optics, Hudson, MA). Chromosomes were analyzed at a rate of 150-300/s in all studies.

Pulse processing electronics

The signals from the photomultipliers were integrated using preamplifiers with a decay time of 100 μs (LEA 75-9044) and amplified with pulse-shaping amplifiers (450 research amplifier, Ortec, EG&G, Oak Ridge, TN) with integration and differentiation time constants of 0.5 μs and 5 μs, respectively. The pulses were digitized with analog to digital converters (Nuclear Data model 572, Schaumberg, IL). These values were accumulated in either 256 channel univariate distributions or 64x64-channel bivariate distributions in a multichannel analyzer (model 620, Nuclear Data).

Curve fitting

The means, areas and coefficients of variation of each peak in representative univariate flow karyotypes were estimated using a computer analysis program (Gray et al., 1975a) in which the sum of N normal distributions was matched to the peaks in the flow karyotype using a least-squares best-fit procedure.

$$SSQ = \sum_{i=1}^{256} \left[y_i - \sum_{j=1}^{N} g(\sigma_j, \mu_j; i) \right] \qquad \text{(Equation 1)}$$

During analysis, the sum of the squares, SSQ, was minimized. N is the number of normal distributions, y_i is the number of counts in channel i of the flow karyotype, and $g(\sigma_j, \mu_j; i)$ is the value in channel i of the jth normal distribution with mean μ_j and standard deviation σ_j. A function approximating the debris continuum was not included, because this continuum was usually negligibly small.

RESULTS

Bivariate analysis of human chromosomes

Figures 7.1-3 show the bivariate HO vs. CA3 fluorescence intensity distributions of chromosomes prepared from three different human cell types. The bivariate distributions were accumulated in a 64 x 64 array. The cell types analyzed include two established cell lines (LLL 811 and LOVO) and one primary culture. The LLL 811 foreskin fibroblasts (figure 7.1) have a high proliferation rate. Cells collected after a colcemid block are primarily metaphase cells. The LOVO cells (figure 7.2) were

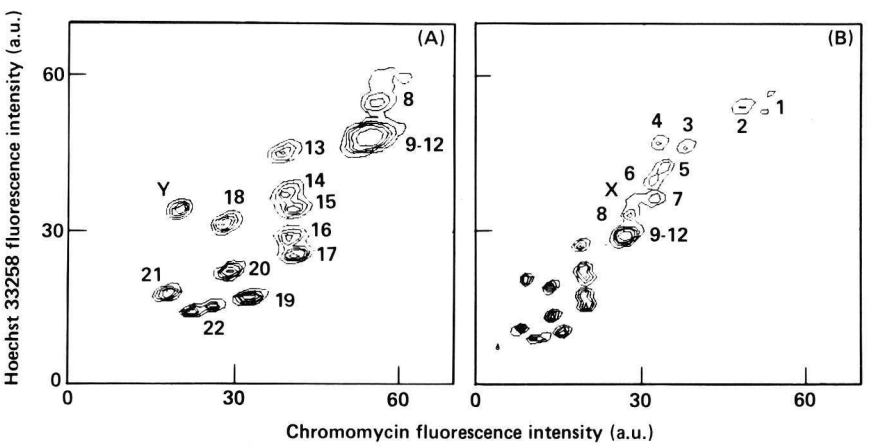


Figure 7.1. Bivariate distribution of Hoechst 33258 vs. chromomycin A3 fluorescence intensity, or flow karyotype, of the chromosomes isolated from human foreskin fibroblasts (LLL 811 cell strain). All chromosomes are displayed in the right panel (B). The left panel (A) shows a distribution generated following adjustment of the amplifier gains so that only chromosomes 8 through 22 and Y are displayed. The chromosomes that are responsible for each peak are indicated by the numbers near each peak. Chromosome identification was determined from previous work in which chromosomes in each peak were sorted, banded, and identified (Gray et al., 1979b; Yu et al., 1981). Chromosomes 1 and 22 are resolved into two homologs. The peaks representing chromosomes X and Y show that only one copy of these two chromosomes is present in these cells. Contour lines are drawn at 10%, 20%, 30%, 40% and 60% of the maximum peak value in the distribution.

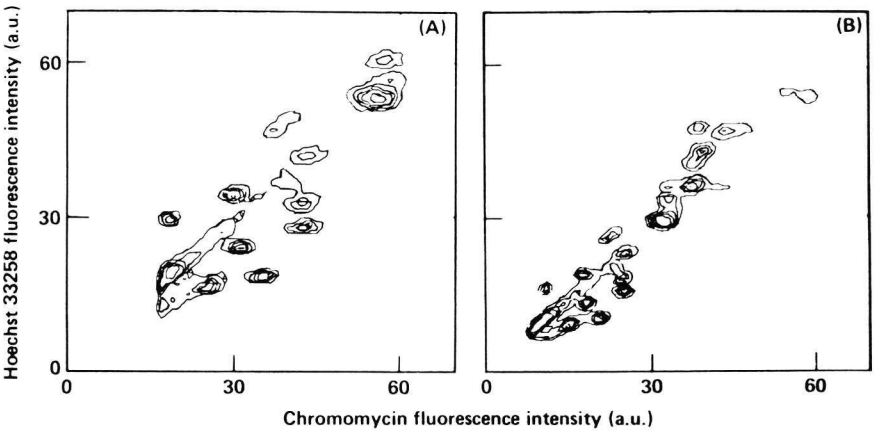


Figure 7.2. Bivariate distribution of Hoechst 33258 vs. chromomycin A3 fluorescence of the chromosomes of a human colon tumor cell line (LOVO). All chromosomes are displayed in the right panel (B). The amplifier gains in the left panel (A) were adjusted to display the smaller chromosomes only. Contour lines as in figure 7.1.

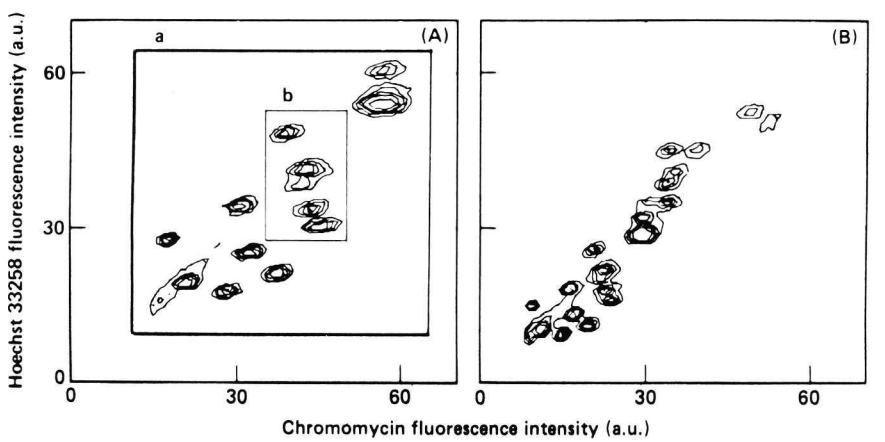


Figure 7.3. Bivariate distribution of Hoechst 33258 vs. chromomycin A3 fluorescence of cells from a phytohemagglutinin-stimulated peripheral lymphocyte culture. The lymphocytes were obtained from a male volunteer. The right panel shows all chromosomes (B). The left panel (A) displays chromosomes 8-22 and Y in detail. Contour lines as in figure 7.1. A univariate HO fluorescence distribution of events falling in the box designated "a" is displayed in the top panel of figure 7.6. A univariate HO fluorescence distribution of events falling in the box designated "b" is shown in the middle panel of figure 7.6.

derived from a human colon carcinoma and are karyotypically instable (Gray et al., 1984). Figure 7.3 shows a flow karyotype measured for chromosomes prepared from a PHA-stimulated lymphocyte culture. Such cultures contain a high percentage of interphase cells (60-90% (Yu et al., 1981)).

The resolution of the peaks in all these distributions is sufficient to resolve most of the human chromosomes. Only the chromosomes 9-12 merge into one peak.

Addition of sodium citrate

The resolution of flow karyotypes can be improved by treating chromosome suspensions with 10 mM Na-citrate, a mild chelating agent. This improvement is most pronounced in the distributions of HO-stained

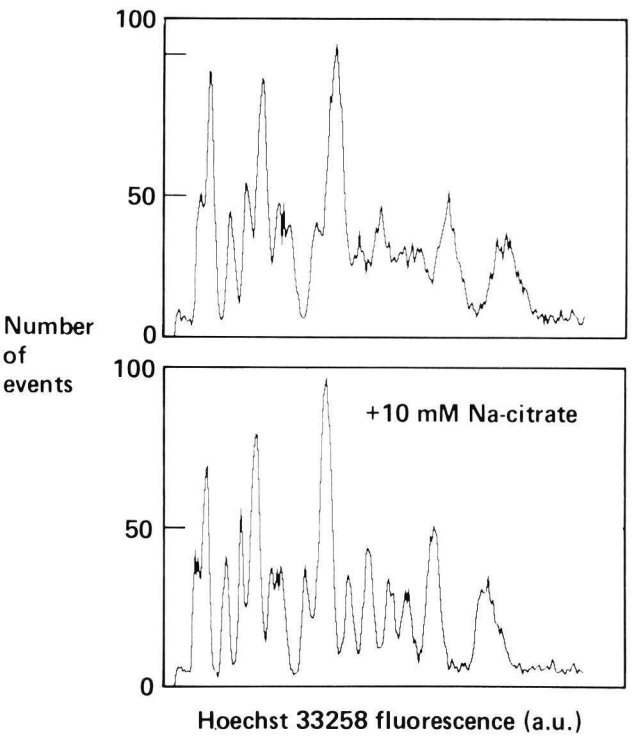


Figure 7.4. Effect of sodium citrate on the univariate distribution of chromosomes stained with Hoechst 33258. A fluorescence intensity distribution of chromosomes prepared from human fibroblast cell line (LLL 811) without the addition of sodium citrate is shown in panel A. Sodium citrate (10 mM) was added 15 min before analysis in panel B. The laser used for UV excitation had a light output of 300 mW.

chromosomes (figure 7.4). Concentrations of Na-citrate lower than 10 mM do not improve the resolution of the distributions. The Na-citrate must be added after the chromosomes have been prepared. If it is added directly to the hypotonic buffer, no chromosomes can be resolved. Once the chromosomes have been obtained in suspension, the Na-citrate does not greatly affect chromosome stability. Excellent flow karyotypes can be obtained from chromosomes stored for several days in the presence of Na-citrate. EDTA, a stronger chelating agent than Na citrate, does not improve chromosome preparations. The addition of 5 mM EDTA destabilizes the chromosomes and causes a rapid decrease in the resolution of the flow karyotype.

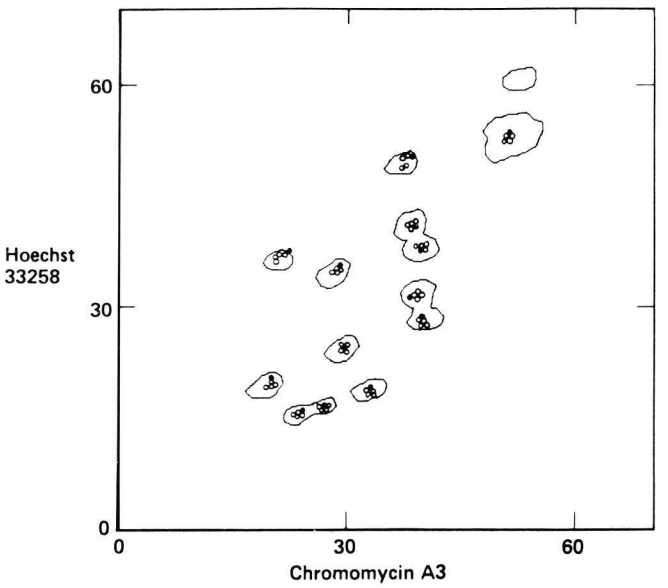


Figure 7.5. Contour lines indicate the bivariate Hoechst 33258 vs. chromomycin A3 fluorescence distribution of chromosomes prepared from approximately 10^5 cells from the LLL 811 human fibroblast cell line measured in the presence of 10 mM sodium citrate. Dye concentrations: 2.7 μM HO, 27 μM CA. UV laser output: 1 W. The lines are drawn at 20% maximum peak value. The solid dots represent the relative peak positions in a flow karyotype of LLL 811 cells collected 2 mo earlier and measured with the same dye concentration, but in the absence of Na-citrate. The open circles represent the relative peak positions in flow karyotypes of chromosomes stained with four HO and CA3 concentration combinations (HO 2.7 μM/CA3 5.6 μM, HO 1.4 μM/CA3 11 μM, HO 5.6 μM/CA3 11 μM, HO 2.7 μM/CA3 23 μM). The UV laser output during the measurement of the flow karyotypes used in determination of the dots and circles was 400 mW. Normalization of the flow karyotypes was performed by determining chromosome peak position by hand and by calculating the average peak position of chromosomes 9 through 22 plus Y for each karyotype. Flow karyotypes measured at different dye concentrations or times were then normalized with respect to the same average peak position.

The coefficients of variation of the peaks of HO-stained human chromosomes treated with Na-citrate are about 2% for the smaller human chromosomes. The resolution of these flow karyotype measurements is largely determined by the statistical variation in the number of photons measured for each chromosome. When the fluorescence signal of HO is reduced 75% by insertion of a 450 nm band pass filter in front of the photomultiplier, the effect of Na-citrate can not be observed.

Flow karyotype reproducibility

The bivariate flow karyotypes shown in figures 7.1 through 7.3 show distinct patterns that are unique for different individuals. If these patterns are sufficiently reproducible, they are potentially useful in the detection of aberrant chromosomes. Our results suggest that these distributions are highly reproducible, even when the chromosome isolation conditions (e.g. stain concentration and Na-citrate presence) are varied. Figure 7.5 shows a contour plot of the flow karyotype for human chromosomes 8 through 22 plus Y isolated from the fibroblast cell strain LLL 811, stained with HO plus CA3 and treated with Na-citrate. The solid dots indicate the relative peak positions of a flow karyotype of the same cell strain measured 2 mo earlier without Na-citrate. The open circles show the relative peak positions in flow karyotypes prepared with concentrations of HO varying from 1.4 μM to 5.6 μM and concentrations of CA3 varying from 5.6 μM to 23 μM. It is evident that the relative peak positions do not change substantially upon treatment with Na-citrate or with changes in dye concentration.

Peak analysis

The detail that can be observed in the bivariate distributions shown in figures 7.1 through 7.3 is limited by the 64 x 64-channel array that is used to display the data. A more accurate representation of peak position and coefficients of variation of closely spaced peaks is obtained when selected areas of the flow karyotype are displayed in a 256-channel univariate distribution. This can be done by measuring the HO distribution of chromosome groups selected by their CA3 fluorescence. This approach also facilitates quantitative analysis of peak means and areas. To see how gated univariate analysis helps, consider first the univariate HO distribution in figure 7.6A. This is the HO distribution for the human lymphocyte chromosomes in region a in figure 7.3 (chromosomes 8 through 22 plus Y). The HO distribution for the chromosomes falling in region b in figure 7.3 (chromosomes 13 through 17) is shown in figure 7.6B. Figure 7.6C shows the gated HO distribution for chromosomes 13 through 17 from the human fibroblast strain LLL 811. There is more detail in figures 7.6B and

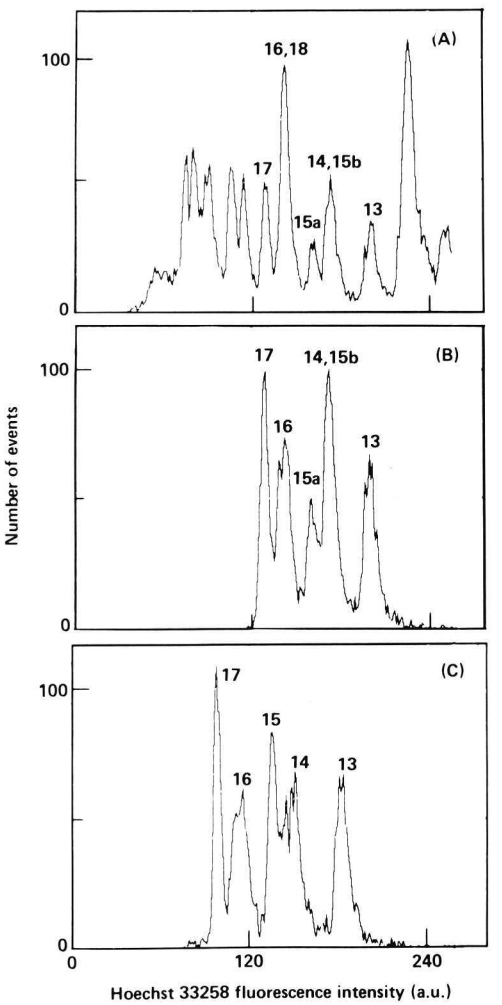


Figure 7.6. Univariate Hoechst 33258 fluorescence intensity distributions of human chromosomes stained with HO and CA3. The HO fluorescence intensity was analyzed only for those events falling into predetermined limits. In the top panel (A), the HO fluorescence intensity of chromosomes 8-22 isolated from the peripheral lymphocyte culture analyzed in figure 7.3 is shown. Small fluorescent particle debris was gated out on the basis of CA3 fluorescence (box a in figure 7.3). The middle panel (B) shows the HO fluorescence distribution of chromosomes 13-17 of the same cells. These chromosomes were selected by analyzing only those chromosomes falling in box b in figure 7.3. The bottom panel (C) shows chromosomes 13-17 of the LLL 811 human foreskin fibroblast cell line selected in a similar manner. The amplification of the HO fluorescence signal in the top two panels is different from that in the bottom panel. The chromosomes responsible for each peak are indicated by the numbers near each peak. Table 7.1 lists the peak means and relative areas of the various peaks in panels B and C.

7.6C than that apparent from the bivariate plots of these cells. Note that in both cell types, homolog differences in chromosome 16 (and 14 for the LLL 811 strain) are detected in the 256 channel representation.

Furthermore, this gated acquisition procedure alleviates some of the difficulties in estimating peak volumes and positions. Mathematical analysis of the distribution in figure 7.6A is complicated by the large number of peaks that must be analyzed simultaneously and by the fact that some chromosomes are not resolved (e.g. chromosomes 16 and 18; 17 and Y). Figure 7.6B, on the other hand, is easier to analyze since fewer peaks are present. Chromosomes 18 and Y do not contribute to this distribution and do not overlap with peaks 16 and 17 as they do in figure 7.6A. Estimation of areas for the peaks in the distributions in figures 7.6B and 7.6C by least-squares best-fit analysis using Equation 1 is straightforward using previously reported computer techniques (Gray et al., 1975). Table 7.1 shows the relative peak areas estimated by this procedure and compares these to the areas expected from conventional karyotyping.

Table 7.1

peak identification	calculated frequency[a]	expected frequency	peak coefficient of variation (%)[b]
Lymphocyte chromosomes			
17	1.9	2.0	2.0[c]
16	2.5	2.0	--[c]
15A	1.0	1.0	1.9
14, 15B	2.8	3.0	2.4
13	1.7	2.0	1.9
Fibroblast chromosomes			
17	1.9	2.0	2.2[c]
16	2.3	2.0	--[c]
15	1.7	2.0	2.0[c]
14	2.4	2.0	--[c]
13	1.7	2.0	2.0

[a] Note that when the calculated frequencies are constrained to be integers, the calculated frequencies agree perfectly with the expected frequencies.
[b] Coefficient of variation calculated as described by Gray et al. (1975b).
[c] Coefficient of variation not calculated due to obvious heterogeneity in the staining of chromosomes producing peak (either homolog difference or partial resolution of chromosomes of different types).

Molecular weight determination

The molecular weight of the DNA that could be extracted from the chromosomes isolated by this procedure was determined by agarose gel electrophoresis. Three chromosome preparations were analyzed: 1) chromosomes isolated, but not stained, 2) chromosomes isolated and stained with HO and CA3 and 3) chromosomes isolated, stained with HO and CA3 and sorted on the LLNL high speed sorter (17). The molecular weight of the DNA extracted from the chromosomes ranged from 20 kb to over 50 kb in all cases.

DISCUSSION

A chromosome isolation procedure that was developed using Chinese hamster cells based on stabilization of chromosomes with $MgSO_4$ (chapter 6) is also suitable for human cells. The univariate and bivariate flow karyotypes from human fibroblasts, peripheral blood lymphocytes, and colon carcinoma cells in vitro are of excellent quality. These flow karyotypes have low debris continua in the vicinity of the smaller chromosome peaks owing to small fluorescent particles. The low debris level facilitates the detection of genetic abnormalities of the smaller chromosomes, such as trisomy 21. The preparation technique works well with low cell numbers and can be applied to primary cell cultures. The high quality of chromosome preparations from PHA-stimulated peripheral lymphocyte cultures opens the possibility to apply flow karyotyping to clinical problems.

Chemical analyses show that the DNA and the structural proteins are well preserved during the preparation procedure. The chromosomes contain histones (chapter 12), and DNA fragments ranging from 20 kb to over 50kb can be extracted from the chromosomes. The low debris level may also be helpful in purifying chromosomes by sorting. This reduces the chance that DNA debris from other chromosomes contaminates the sorted fractions of the smallest chromosomes. These features should make the procedure attractive to investigators interested in purification of chromosomes for production of recombinant DNA libraries or for gene localization using hybridization techniques.

The observation that the resolution of the fluorescence distributions can be improved by addition of sodium citrate after the chromosomes have been prepared and stained is of special interest. The mechanism of action of sodium citrate is not well understood; however, it probably involves manipulation of the Mg^{++} concentration of the chromosome isolation buffer. In this procedure, the chromosomes are isolated in an isolation

buffer with a relatively high Mg^{++} concentration to stabilize the DNA of the chromosomes in the absence of intercalating dyes. However, high Mg^{++} concentrations cause chromosome compaction (Marsden and Laemmli, 1979), which may negatively affect the resolution of flow cytometric measurements. The addition of Na-citrate improves the chromosome preparation, perhaps by acting as a buffer of free Mg^{++} ions. Na-citrate is a chelating agent a relatively low binding constant ($k=10^3$), and its presence does not reduce chromosome stability significantly. The fluorescence of chromomycin, a dye that requires Mg^{++} for binding to DNA, can be measured after Na-citrate addition. When a stronger chelating agent such as EDTA ($k=10^{10}$) is added, the chromosomes become unstable and fall apart.

A cause of concern in the bivariate analysis of chromosomes is that changes in the preparation or staining conditions may cause shifts in the relative peak positions of the chromosomes. These shifts are expected if chromosomes with the same adenine-thymidine to guanine-cytosine differ in the efficiency of energy transferred from HO to CA3 (Langlois et al., 1980; Langlois and Jensen, 1979) or if there are two types of HO binding sites (Latt and Wohlleb, 1975). The addition of Na-citrate and fourfold variations in HO or CA3 concentrations do not significantly affect the relative peak positions of the chromosomes. The addition of Na-citrate affects the absolute fluorescence intensity of HO- and CA3-stained chromosomes, yet the relative peak positions are unaffected. This indicates that differing energy transfer efficiencies among chromosomes with the same HO/CA ratio are of minor importance in flow karyotype measurements. However, when the conditions are varied over a wider range, such as a 25-fold increase in cell concentration or a 16-fold range in stain concentration, some changes in the position of some chromosomes may occur. These findings will be addressed in more detail in chapter 8.

The advantage of bivariate analysis over single parameter fluorescence distributions is obvious when figures 7.1 and 7.4 are compared. Combined staining with HO and CA3 allows discrimination between chromosomes that overlap when either of the two dyes is used alone. In the combined staining, more peaks are resolved along the HO axis than along the CA3 axis. The distributions of figure 7.6 show that the presence of CA3 does not reduce the detail that can be observed in the HO profiles. However, some resolution is lost, especially along the HO axis, when data are accumulated in a 64 x 64-channel bivariate distribution. Sixty-four channels are not sufficient to resolve all of the peak structure that is potentially available. Similar findings have been reported by Lebo and Bastian (1982). This loss of univariate resolution is acceptable because of the tremendous gain in peak resolution resulting from the measurement of

two variables per chromosome. By using gated acquisition of selected peaks in the bivariate distribution, full HO resolution can still be obtained and utilized.

Chapter 8

THE INTERACTION AND BINDING KINETICS OF DNA FLUOROCHROMES USED IN FLOW KARYOTYPING

SUMMARY

The interactions and binding characteristics of DNA dyes used in flow cytometric analysis of chromatin were studied using chromosomes and mouse thymocyte nuclei. The kinetics of dye binding and the relationship between fluorescence intensity and dye concentration are presented. Under the conditions used, propidium iodide, Hoechst 33258 and chromomycin A3 reach an equilibrium with thymocyte nuclei after approximately 20 min, 5 min and more than 1 h, respectively. The same binding kinetics are observed with the two latter dyes when nuclei are stained with a mixture of Hoechst and chromomycin. Sodium citrate, which improves the resolution of flow karyotypes, causes a rapid increase in Hoechst and propidium iodide fluoresence, but a decrease in the fluorescence of chromomycin. The relative peak positions of chromosomes in a flow karyotype are unaffected by sodium citrate addition. The spectral interaction between Hoechst and chromomycin is quantified. There is variation among the human chromosome types in the amount of energy transferred from Hoechst to chromomycin. By measuring the Hoechst and chromomycin fluorescence of each chromosome after Hoechst excitation, it is shown that the amount of energy transferred is correlated to the ratio of the amount of Hoechst to chromomycin bound. Although the energy transfer between the two dyes is considerable, this has little effect on the reproducibility of flow karyotype measurements. The relative peak positions of all human chromosomes except number 13 and Y vary in the order of 0.5 channel over a 16-fold change in either Hoechst or chromomycin concentration. This implies that, with the present flow karyotype machines, variation in staining conditions will have minimal effects on the reproducibility of the relative peak positions in flow karyotypes.

INTRODUCTION

One important potential application of flow karyotyping is the detection of aberrations that occur in most or all of the cells of a population. In this application, the aberrant chromosomes are detected

because their peak position in a bivariate flow karyotype deviates from that of the normal chromosomes. The sensitivity of bivariate flow karyotyping in detecting these shifts in peak position depends on the variability in the location of the normal peaks from sample to sample and from person to person. Langlois et al. (1982) investigated the variation in relative peak positions in flow karyotypes measured for chromosomes isolated from peripheral lymphoctyes from normal individuals. That study showed that the variability in flow karyotypes among individuals is larger than the variability among replicate measurements on the same individual. The variability among individuals was also shown to be sufficiently small, that most of the chromosomes in a flow karyotype can be consistently identified solely on the basis of peak position. Rearrangements in chromosomes as small as a single band should be detectable. These findings suggest that flow karyotyping may achieve a level of sensitivity in the detection of chromosomal abnormalities approaching that of the traditional analysis of banded chromosomes.

Despite these promising findings, the potential variability in flow karyograms due to variations in the preparation procedure is a cause of concern for any chromosome isolation procedure. Flow karyotype resolution is dependent on homogeneous staining of identical particles. For highest chromosome resolution, chromosomes should be well equilibrated with optimal concentrations of fluorescent dyes. Since the chromosomes are measured in the presence of free dye, the dyes are often employed at less than saturating concentrations. The interaction of dyes used in bivariate chromosome analysis may also contribute to flow karyotype variability. CA3, a DNA ligand specific for guanine-cytosine (G-C) base pairs, and HO, a dye that binds preferentially to adenine-thymidine (A-T) base pairs, are the two stains most commonly used for bivariate flow karyotyping. These fluorescent dyes overlap in their spectral properties (Langlois and Jensen, 1979; Latt et al., 1980). There is a high probability of resonance energy transfer from HO to CA3 when they bind to DNA. Since resonance energy transfer is highly dependent on the distance between donor and acceptor molecules, the efficiency of energy transfer is affected by the concentration and distribution of bound dyes. Latt and Wohlleb (1975) reported the presence of two HO-binding sites in DNA, the second being filled at high HO concentrations. Langlois et al. (1980) observed shifts in the relative peak positions of some singly-stained chromosomes with changes in HO concentration. These shifts were attributed to differences among chromosomes in the relative number of the two HO-binding sites. These authors also observed that some chromosomes exhibited less energy transfer than that expected from the ratio of HO to CA3 fluorescence. This deviation was ascribed to different distributions of

A-T and G-C base pairs in chromosomes with the same A-T/G-C ratio. If these phenomena indeed cause substantial variation in flow karyotypes, interpretation of flow karyotypes of chromosomes prepared using different staining protocols, from varying numbers of cells, or with different dye concentrations could be difficult or impossible.

This chapter describes an evaluation of the extent to which variations in dye concentration and staining time influence flow cytometric measurements of nuclei or chromosomes isolated using the procedure described previously (chapter 6, 7). Mouse thymocyte nuclei are used as a model system to study dye uptake and binding characteristics. The observations reported here result in guidelines to minimize flow karyotypic variability due to non-equilibrated staining and due to variations in dye ratios and concentrations.

MATERIALS AND METHODS

Isolation of chromosomes

Chromosomes were isolated from cells of the male human diploid fibroblast cell strain (LLL-811) derived from foreskin as described previously (chapter 7).

Thymocyte nuclei

Thymocytes were obtained from 15 wk-old male C3H/HE mice by releasing the contents of the thymus into phosphate buffered saline, containing Ca^{++} and Mg^{++}. The cell suspension was filtered through nylon gauze and centrifuged at 100g for 10 min. Nuclei were isolated from the cells in the resulting pellets using the same procedure as that for chromosome isolation. The cells were resuspended in isolation buffer at a concentration of $2x10^6$ cells/ml. After the addition of Triton X-100, suspension was vortexed vigorously for 10-20 s in place of syringing through a needle.

Staining

The stock solutions of the DNA dyes were made in distilled water and filtered through a 0.22 µm Millex GS filter unit (Millipore, Bedford, MA) before use. Stock concentrations were 210 µM Hoechst 33258 (HO), 525 µM chromomycin A3 (CA3), 570 µM propidium iodide (all from Sigma). For full equilibration, chromosomes or thymocytes were incubated in the DNA dyes for at least 2 h before analysis.

In some cases, sodium citrate (Na-citrate) was added to a final concentration of 10 mM, at least 2 h after staining and 15 min before flow analysis.

Flow cytometric analysis

Flow cytometric analysis was performed on the LLL dual beam flow cytometer equipped with two lasers as described in chapter 7. CA3 was excited at 458 nm (200-300 mW laser power), and the resulting fluorescence was measured through a 500 nm long pass filter (3-71, Corning). In the text, this fluorescence is referred to as CAfl:CAex. HO was excited in the ultraviolet range (multiline, 334, 351 and 363 nm) (laser power as indicated in figure legends), and the resulting fluorescence was measured through a UV blocking filter (425 high pass, Ditric Optics, Hudson MA). This is referred to as HO fluorescence, but is a composite of the light emitted directly from HO and the light emitted from CA3 after resonance energy transfer from HO (HOfl:HOex+CAfl:HOex). A laser tuned to emit light at 488 nm at 800 mW output was used to excite PI. PI fluorescence was measured after passing through a 600 nm long pass filter (Corning 2-60). Events were analyzed at a rate of 200-500 per second.

RESULTS

The binding of DNA fluorochromes to chromatin was studied using nuclei from mouse thymocytes. The nuclei were prepared using the same procedures that are used to isolate chromosomes from mitotic cells. Thymocytes have a low proliferation rate, so that their DNA distribution consists almost

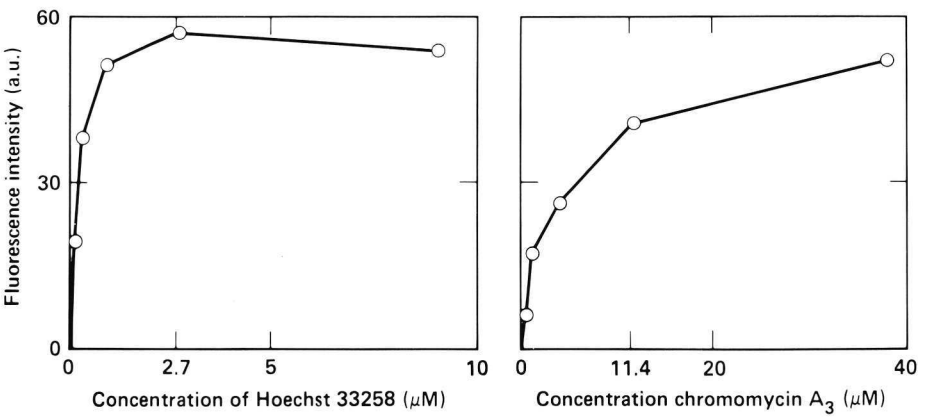


Figure 8.1. Effect of dye concentration on the fluorescence intensity of thymocyte nuclei. Nuclei were incubated in the indicated concentration of HO (left panel) or CA3 (right panel) for 2 h before the peak channel position of the fluorescence intensity distribution was determined. Amplifier gains and photomultiplier settings were constant for the measurements in each panel. Laser output for UV excitation in this experiment was 500 mW; for 458 nm, it was 300 mW.

entirely of a single peak of G_0/G_1 cells. The position of this this single narrow peak can be determined more accurately in a fluorescence distribution than when a family of peaks is present, as measured with a suspension of chromosomes. This is especially true at suboptimal staining conditions, which may result in broadened peaks.

Figure 8.1 shows the fluorescence intensities of nuclei incubated with varying concentrations of HO or CA3. The fluorescence distributions were measured after the nuclei had been equilibrated with the dyes for at least 2 h. The intensity of staining with HO saturates at approximately 2.7 μM. In the concentration range tested here, no saturation of CA3 fluorescence was observed. Peaks in the CA3 fluorescence distributions become broader at higher CA3 concentrations. Concentrations of 2.7 μM HO and CA3 ranging from 10-30 μM have been shown previously to result in optimum resolution of individual chromosome types in bivariate human flow karyotypes (chapter 7).

Simultaneous staining of thymocyte nuclei with HO and CA3 reveals interaction between the two dyes. The HO fluorescence intensity of nuclei equilibrated in a mixture of HO (2.7 μM) and CA3 (11 μM) is 48% less than that of nuclei stained with HO alone. The CA3 fluorescence intensity of nuclei stained with both HO and CA3 is only 5% less than that of nuclei stained with only CA3. Chromosomes stained with both dyes exhibit similar reductions in the fluorescence intensity of HO and CA3 (data not shown).

Effect of sodium citrate addition

The coefficient of variation (CV) of the peaks in a fluorescence distribution can be decreased by the addition of 10 mM sodium citrate (Na-citrate) to stained nuclei or chromosomes before analysis (chapter 7). This reduction is most pronounced with HO-stained chromosomes and is accompanied by changes in absolute fluorescence intensity. Table 8.1 summarizes the effect of Na-citrate addition on nuclei or chromosomes stained with three fluorescent DNA stains. The fluorescence intensity of nuclei stained with either PI or HO increases considerably upon Na-citrate addition. The CA3 fluorescence intensity decreases by 21%. When nuclei or chromosomes are stained with both HO and CA3, the reduction in CA3 fluorescence intensity when Na-citrate is added is also approximately 20%. The increase in HO fluorescence after Na-citrate addition is approximately 2 times greater in nuclei stained with both HO and CA3 than in nuclei stained with only HO. Doubly-stained chromosomes show the same relative increase in HO fluorescence and decrease in CA3 fluorescence upon Na-citrate addition as thymocyte nuclei do. Although this absolute fluorescence intensity change occurs of chromosomes treated with Na-citrate addition, the relative peak positions of chromosomes in a flow karyotype remain the same (see below).

The effect of Na-citrate on fluorescence intensity begins almost immediately after the addition of Na-citrate to a suspension of nuclei or chromosomes and is complete within approximately 5 minutes.

The kinetics of DNA staining

The kinetics of the binding of DNA dyes were determined by mixing unstained thymocyte nuclei with nuclei that had been equilibrated for at least 2 h with a DNA dye. The stained nuclei serve as an internal control. Immediately after mixing, the dye concentration was adjusted for the volume increase resulting from addition of the unstained cells. A fluorescence intensity distribution of the mixture was measured after different time intervals. The position of the peak representing the unstained nuclei can

Table 8.1

EFFECT OF 10 mM Na-CITRATE ON THE FLUORESCENCE INTENSITY OF MOUSE THYMOCYTE NUCLEI OR HUMAN CHROMOSOMES STAINED WITH DNA-SPECIFIC DYES

stain (concentration)	fluorescence intensity change after Na-citrate (% increase in peak position)	coefficient of variation (%) before Na-citrate	coefficient of variation (%) after Na-citrate
Nuclei stained with:			
Propidium iodide	+10	2.3	1.5
Hoechst 33258	+22	1.0	0.8
Chromomycin A3	-21	1.6	1.2
Hoechst 33258 plus Chromomycin A3	+43 -20		
Chromosome stained with:			
Hoechst 33258 plus Chromomycin A3	+41 -21		

Mouse thymocyte nuclei or human chromosomes were stained with the given DNA stain(s). Stain concentrations were 29 µM PI, 2.7 µM HO, and 27 µM CA3. After 2 h at 4°C, the fluorescence intensity distribution was measured. Na-citrate was added to 10 mM final concentration, and the fluorescence intensity distribution was remeasured. The percent difference in the peak channel position of the two distributions is indicated in the second column. The coefficient of variation (CV) of the single peak in the fluorescence distribution for nuclei was calculated with the Livermore program for fitting fluorescence distributions with Gaussian distributions (Gray et al., 1975b).

be accurately determined with respect to the peak representing the nuclei in equilibrium with dye. This experimental approach eliminates possible drifting in the electronics or in laser light output that can occur over long periods of time.

The procedure is illustrated in figure 8.2. The figure shows fluorescence intensity distribution made at four time points after mixing unstained nuclei with nuclei that had been stained with 29 μM PI for over 2 h. The rate of dye binding can be determined by following the movement of the peak of unstained nuclei in time. After the fluorescence intensity distributions of the two populations merge, the width of the single peak

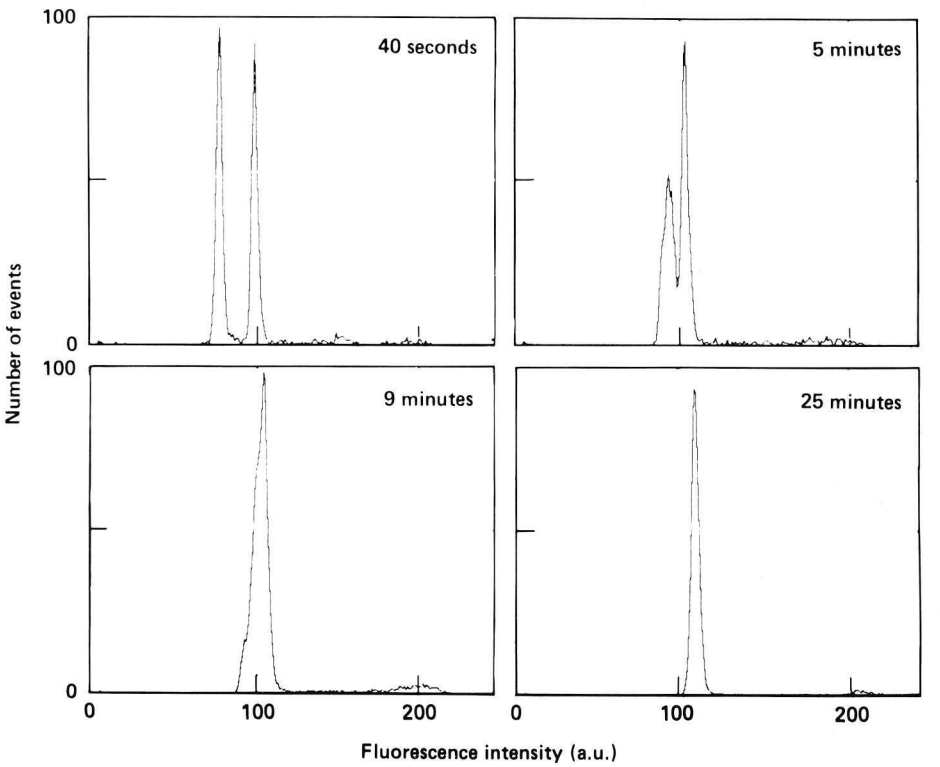


Figure 8.2. Fluorescence intensity distributions of unstained nuclei and nuclei at equilibrium in 29 μM PI at 40 s, 5 min, 9 min, and 25 min after they were mixed together. At time 0, an equal volume of unstained nuclei was added to nuclei in equilibrium with PI, and the PI concentration was corrected to 29 μM. Two peaks are visible in the top two panels. The left peak is produced by the initially unstained nuclei and the right peak is produced by the initially stained nuclei. The rate at which the 2 peaks merge is a measure of the binding rate of PI to DNA.

continues to decrease. When the width of the peak reaches its minimum value, the nuclei can be considered to be completely equilibrated.

The results of such measurements are presented in figures 8.3 and 8.4. Figure 8.3 shows the increase in the propidium iodide fluorescence for thymocyte nuclei in time. After approximately 10 min in PI, the fluorescence intensity of unstained nuclei reaches the level of nuclei equilibrated for 2 h in PI. After the fluorescence intensity distributions of unequilibrated and equilibrated nuclei merge, the CV of the single peak continues to decrease until approximately 20 minutes after the addition of the unstained cells. Figure 8.4 shows the increase in the HO and CA3 fluorescence intensity of unstained thymocyte nuclei in time after staining with either HO or CA3. HO uptake is complete after approximately 5 min. The equilibration of CA3 binding takes much longer to complete. At 25 min,

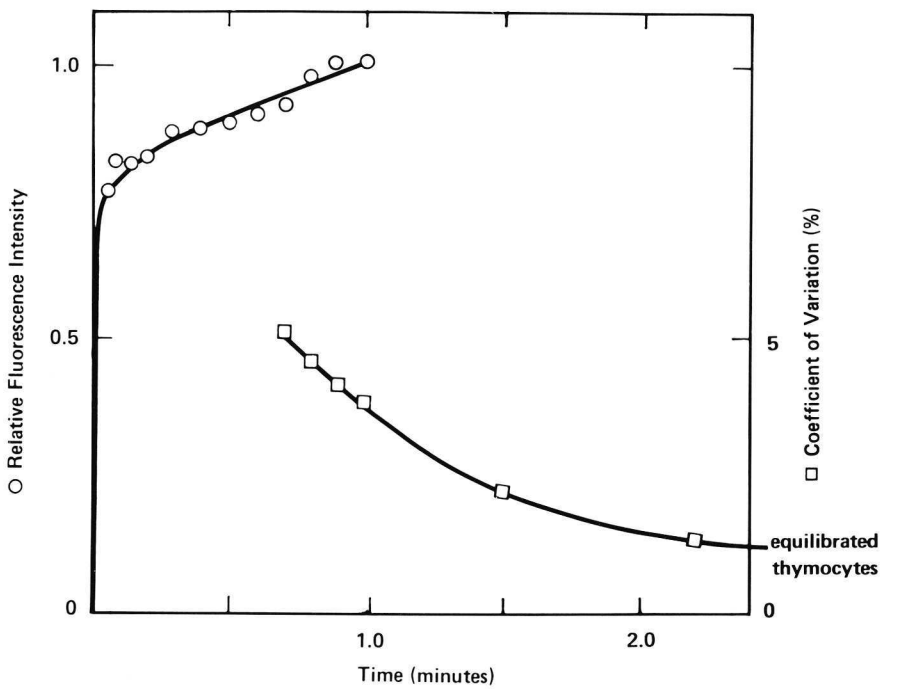


Figure 8.3. The increase in PI fluorescence of unstained thymocyte nuclei after mixing in a PI solution containing nuclei already in equilibrium as described in figure 8.2. At each time point, the peak position of the fluorescence distribution of unequilibrated nuclei was determined relative to the peak position of equilibrated nuclei (-O-). After the fluorescence intensity distributions of unequilibrated and equilibrated nuclei had merged, the coefficient of variation (CV) of the peak continued to decrease (-□-).

the fluorescence intensity of unequilibrated nuclei is still only 80% that of nuclei equilibrated in CA3.

The kinetics of the binding of one DNA dye to nuclei already in equilibrium with a second DNA dye can be determined in a similar manner. For HO and CA3 staining, the peaks of equilibrated and unstained nuclei are followed in time in a series of bivariate fluorescence distributions. The HO fluorescence of the nuclei is indicated on the ordinate, and CA3 fluorescence is indicated on the abscissa. The fluorescence distribution of nuclei that are in equilibrium with both HO and CA3 serves as a reference peak. The change in fluorescence intensity of unstained nuclei or nuclei in equilibrium with one of the dyes can be determined. The peak positions of unstained, CA3-stained or HO-stained nuclei with respect to doubly-stained nuclei are plotted in figure 8.5 to show their movement in a series of bivariate distributions measured at different times as the DNA stain(s) bind to the nuclei. Trace A shows the change in CA3 and HO fluorescence of nuclei at equilibrium in HO, after the addition of CA3. As CA3 binds to the nuclei, the fluorescence of the bound HO is quenched. The HO fluorescence intensity of nuclei equilibrated in 2.7 μM HO decreases 50%

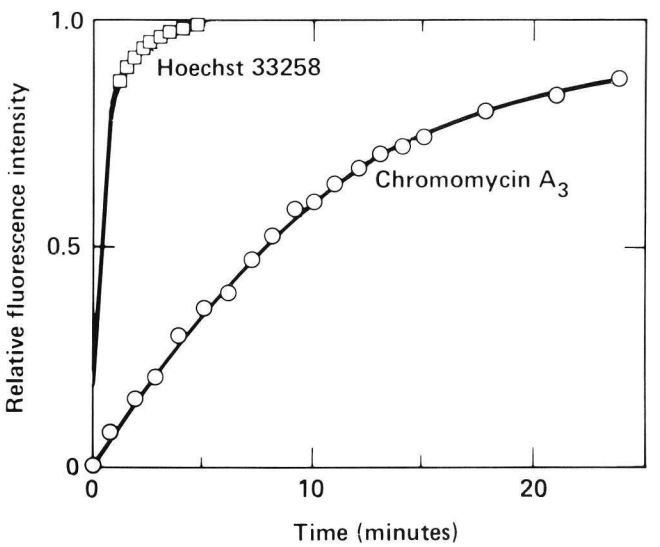


Figure 8.4. The increase in Hoechst 33258 (-□-) or chromomycin A3 (-○-) fluorescence of unstained thymocyte nuclei in time. Uptake was followed using a series of fluorescence intensity distributions of a mixture of unequilibrated nuclei and nuclei in equilibrium with the stain at various time points after mixing, as shown in figure 8.2. At each time point, the peak channel of the distribution of unequilibrated nuclei was determined relative to the peak channel position of dye-equilibrated nuclei. HO concentration: 2.7 μM, CA3 concentration: 11.4 μM. UV excitation was 500 mW and 458 nm excitation was 300 mW.

after the nuclei are exposed to 11.4 μM CA3. Trace B shows the change in the CA3 and HO fluorescence intensity of nuclei in equilibrium with CA3 after the addition of HO. In this case, the CA3 fluorescence decreases by only approximately 5%, while the HO fluorescence increases sharply. Trace C shows the changes in CA3 and HO fluorescence that take place for unstained nuclei after they are added to a solution containing HO and CA3. HO fluorescence intensity increases rapidly initially, only to decrease slowly somewhat later. These observations are consistent with the binding kinetics of HO and CA3 shown in figure 8.4. HO binds rapidly, and the HO

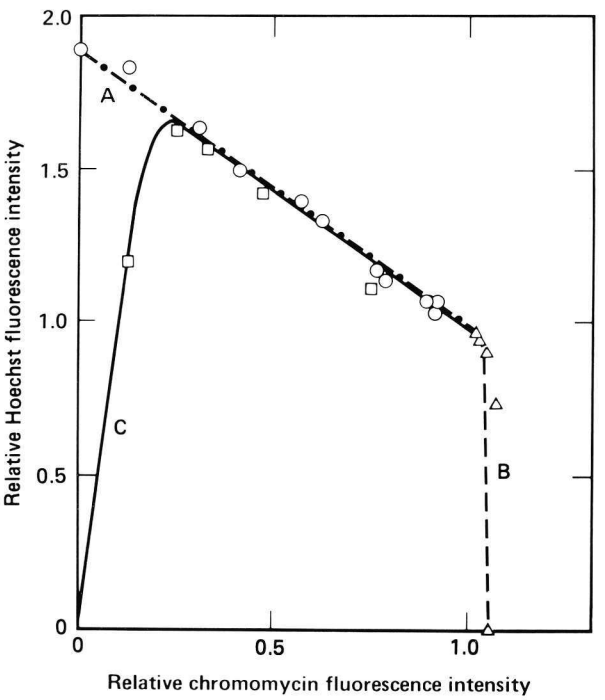


Figure 8.5. The movement of the peak position of thymocyte nuclei as the nuclei equilibrate in HO, in CA3 or in a mixture of the two DNA stains. The peak position was derived from bivariate HO vs. CA3 fluorescence intensity distributions in which both unequilibrated and equilibrated nuclei were measured. At various time points after mixing dye-equilibrated and unstained nuclei together, the peak position of the unequilibrated nuclei was determined relative to the peak position of the nuclei in equilibrium with the dye. Trace A (O) shows the peak position movement of nuclei equilibrated in 2.7 μM HO after exposure to CA3 (11.4 μM). Trace B (Δ) shows the movement of nuclei equilibrated in CA3 after mixing with HO. Trace C (□) shows the movement of the peak position of unstained nuclei after the simultaneous addition of HO and CA3. The time scales of traces A and B are shown in figure 8.6. UV excitation was 400 mW and 458 nm excitation was 300 mW.

fluorescence increases accordingly. CA3 binds more slowly to nuclei. As the amount of CA3 bound to the nuclei increases, the HO fluorescence is quenched and slowly decreases. The time scale in which these changes take place is shown in figure 8.6. The binding rates of HO and CA3 to nuclei that are already in equilibrium with the other dye are similar to the binding kinetics of HO or CA3 to unstained nuclei (figure 8.4).

Energy transfer between HO and CA3

HO fluorescence was observed in the experiments above to decrease approximately 50% in the presence of CA3. The two dyes exhibit minimal binding competition (trace B, figure 8.5). The reduction of HO fluorescence can be ascribed to resonance energy transfer to CA3. This transferred energy may in turn be emitted as CA3 fluorescence. In this section, experiments are described which were designed to provide an estimate of the relative contribution of CA3 fluorescence to total fluorescence after HO excitation.

In a two laser flow system, the following fluorescence signals can be recognized: the HO fluorescence after HO excitation (HOfl:HOex), the CA3

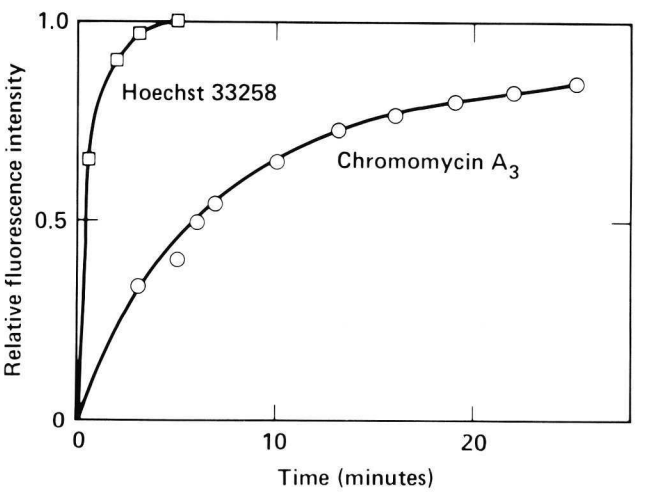


Figure 8.6. The change in time of the HO fluorescence (-□-) of thymocyte nuclei that were in equilibrium with CA3 (11.4 μM), after mixing them with HO (2.7 μM) at time 0. The change in time of the CA3 fluorescence (-○-) of thymocyte nuclei at equilibrium in HO, after mixing them with CA3 at time 0. In both cases, the fluorescence intensity of the unequilibrated nuclei was determined at each time point relative to the peak position of nuclei at equilibrium with both HO and CA3. Laser excitation as in figure 8.5. The measurements for this figure differ from those for figure 8.4 only in that the nuclei for figure 8.4 were unstained before the addition of HO or CA3.

fluorescence after HO excitation (CAfl:HOex), and the CA3 fluorescence after CA3 excitation (CAfl:CAex). Conventionally (and in the measurements reported above), the "HO fluorescence" is the fluorescence after UV excitation and is measured through a UV-blocking (425 nm high pass) filter. For doubly-stained chromosomes the measured fluorescence is a composite of the fluorescence spectra of both HO and CA3 (HOfl:HOex + CAfl:HOex).

An estimate can be made of the contribution of HOfl:HOex and CAfl:HOex to the total fluorescence after UV excitation by inserting a 450 nm band pass filter in front of the photomultiplier measuring the fluorescence upon HO excitation. This filter blocks all of the CA3 fluorescence. No appreciable fluorescence signals are observed through this filter for chromosomes stained only with CA3. The 450 nm band pass filter also blocks a portion of the HO fluorescence. The transmission factor of this filter for the HO fluorescence spectrum can be determined by measuring the reduction in the signal intensity measured of HO-stained nuclei caused by

Table 8.2

EFFECT OF FILTER COMBINATIONS ON THE MEASURED FLUORESCENCE INTENSITY OF HO- OR (HO+CA3)-STAINED THYMOCYTE NUCLEI

stain	measured light intensity through		calculated light intensity with	
	425 high pass A	450 wide band B	HO spectrum C	CA3 spectrum D
Without sodium citrate				
HO	100	17	100	0
HO + CA3	52	6	35 (6x100/17)	17 (52-35)
With sodium citrate				
HO	100	17	100	0
HO+CA3	61	9	53 (9x100/17)	8 (61-53)

Fluorescence distributions were measured for mouse thymocyte nuclei, stained with 2.7 μM HO or with a combination of HO and 27 μM CA3, and treated with or without Na-citrate. Fluorescence intensity values of nuclei are normalized to the intensity of nuclei stained with only HO and measured after UV excitation through a 425 high pass filter. The intensity given in column A is a measure of the fluorescence of both HO and CA3 after excitation of HO (HOfl:HOex + CAfl:HOex). Column B indicates the intensity of fluorescence measured through a filter (450 nm band pass). This filter blocks all CA3 fluorescence and passes only a portion (17%) of the HO fluorescence. The fluorescence indicated here is a measure HO fluorescence after HO excitation (HOfl:HOex). In column C, the HO fluorescence intensity after HO excitation is corrected for the effect of the 450 wide band filter (x100/17). In column D, the intensity of CA3 fluorescence after HO excitation (CAfl:HOex) resulting from resonance transfer from HO to CA3 is calculated by subtracting the value in column C from that in column B.

insertion of the filter. Table 8.2 summarizes the effect the 450 nm BP filter on the fluorescence intensity measured of nuclei (columns A vs. B). Measurements were made both in the presence and absence of Na-citrate and are presented separately in the table. In each case, the measured fluorescence intensities are normalized with respect to the fluorescence of HO-stained nuclei measured through the UV blocking filter (425 nm HP) only. Insertion of the 450 nm BP filter reduces the signal from HO-stained nuclei stained by 83%. Similar reduction is observed in the presence of Na-citrate. The portion of a fluorescence signal that can be ascribed to HO fluorescence (the HOfl:HOex component) can, therefore, be calculated by multiplying the signal measured through a 450 nm BP filter by 100/17. Column C of table 8.2 presents the intensities of the HOfl:HOex calculated in this manner for thymocyte nuclei stained with HO only or with HO and CA3. The figures in column A represent the combined fluorescence intensity of HOfl:HOex plus CAfl:HOex. Therefore, the CAfl component of the total fluorescence after HO excitation can be calculated by subtracting the values in column C from those in column A. These data indicate that approximately 33% of the fluorescence after HO excitation of doubly-

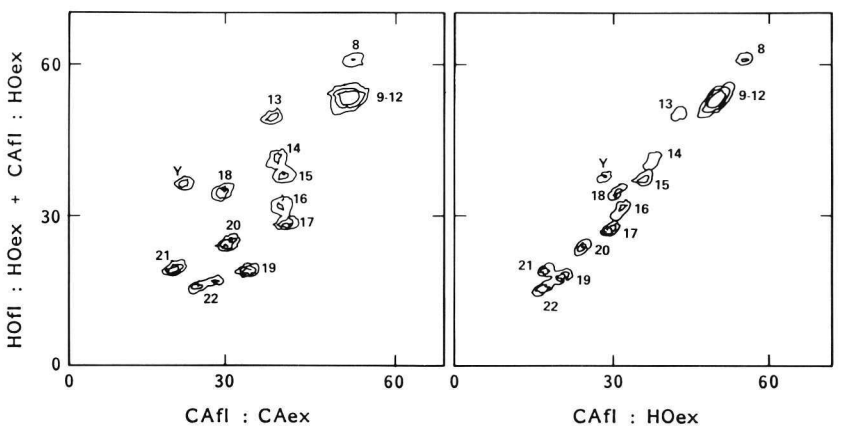


Figure 8.7. Bivariate fluorescence intensity distributions of human chromosomes 8-22 and Y stained with HO (2.7 μM) and CA (27 μM). In the left panel (A), the "HO fluorescence", the intensity of fluorescence with wavelengths greater than 425 nm after UV excitation (HOfl:HOex+CAfl:HOex), is plotted on the ordinate; the intensity of fluorescence after excitation at 458 nm (CAfl:CAex) is plotted on the abscissa. In contrast, the abscissa in the right panel (B) represents CA3 fluorescence (wavelengths greater than 500 nm) after UV excitation (CAfl:HOex). The ordinate in the right panel is again (HOfl:HOex+CAfl:HOex) as in the left panel. The identity of the chromosomes responsible for the peaks in the right panel was determined by measuring the distribution in the right panel for peaks selected in the left panel. UV excitation was 1 W, and 458 nm excitation was 250 mW.

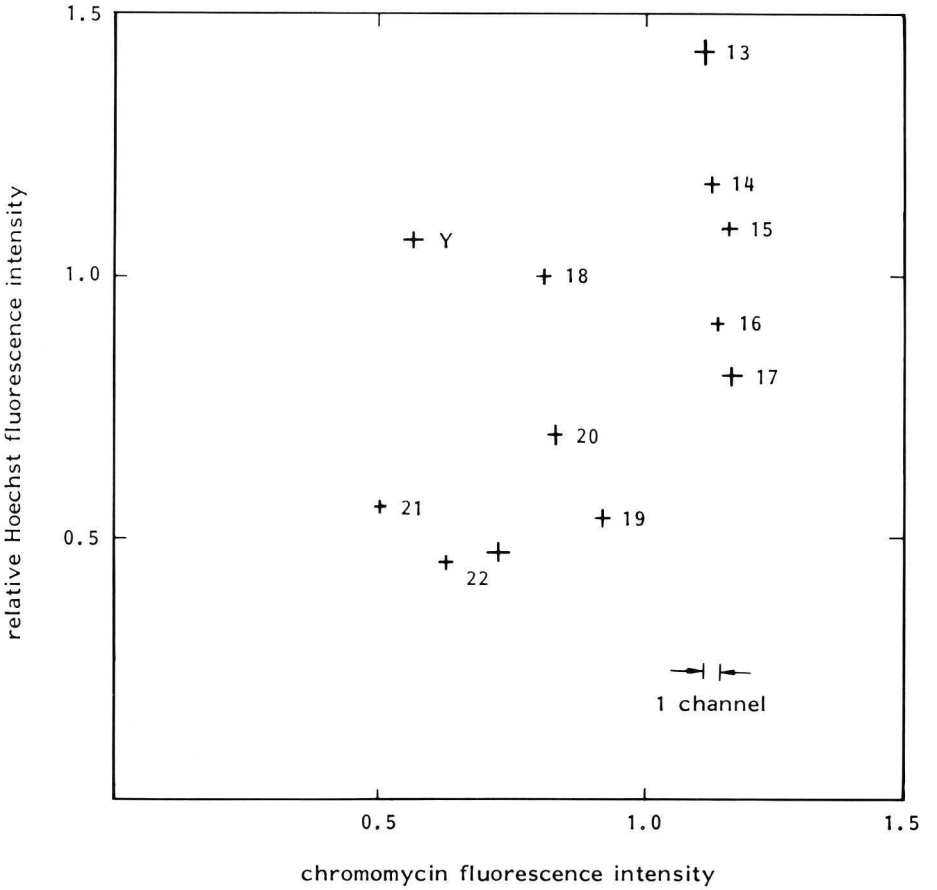


Figure 8.8. Variation in the relative peak positions of chromosomes from the same cell line with 16-fold variations in HO and CA3 concentration. The chromosomes were isolated from the LLL 811 human fibroblast cell line. The concentration of HO was varied using two-fold dilution steps from 11-0.7 μM at a constant CA3 concentration of 11.4 μM. The CA3 concentration was varied using two-fold dilution steps from 46-2.9 μM at a HO concentration of 2.6 μM. All samples were tested with and without sodium citrate addition. Measurements made over a period of 2 mo are included, and they reflect the karyotype of this cell line at various passages. HO fluorescence is a measurement of fluorescence with wavelengths greater than 425 nm after UV excitation (400 mW); CA3 fluorescence is fluorescence greater than 500 nm after excitation with 458 nm light (300 mW). The karyotypes were normalized by determining the chromosome peak position by hand and by calculating the average peak position of the chromosomes 8-22 +Y for each karyotype. Flow karyotype measurements at different dye concentrations were then normalized with respect to the same average peak position. The cross indicates the range of variation in the relative peak position of each chromosome. The width of one channel in the 64 x 64 matrix of the pulse height channal analyzer is indicated in the lower right corner.

stained nuclei has the CA3 fluorescence spectrum. In the presence of Na-citrate, the contribution of the CA3 fluorescence is reduced to 13%.

The HO fluorescence, as well as the CA3 fluorescence, after HO excitation can be determined for chromosomes in suspension. The right panel in figure 8.7 shows the bivariate fluorescence distribution for a suspension of human chromosomes stained with HO and CA3. On the ordinate is the total fluorescence after HO excitation, on the abscissa the CA3 fluorescence after HO excitation. Only chromosomes 8-22 and Y are shown. This distribution can be compared to the conventional bivariate fluorescence distribution in the left panel of figure 8.7. Here, the ordinate represents the fluorescence after HO excitation (HOfl:HOex+CAfl:HOex), and the abscissa indicates the CA3 fluorescence after excitation at 458 nm (CAfl:CAex). The chromosomes responsible for the peaks in the right panel were identified by electronically gating each peak in the left panel and plotting these selected events in the right panel.

Effect on flow karyotypes

The observations reported above show that a significant amount of energy transfer occurs in chromosomes stained with both HO and CA3. In theory, this may imply that the relative peak position of the chromosomes is dependent on the absolute and relative concentrations of HO and CA3. The actual extent of this effect was studied by evaluating the variability in relative peak position in flow karyotypes measured for one cell line at different concentrations of HO and CA3. For each flow karyotype measured, the peak position of each chromosome type was determined by hand, and the average peak position for each karyotype was calculated. The chromosome peaks in the various flow karyotypes were then normalized with respect to the same average peak position. These measurements are summarized in figure 8.8. Flow karyotypes made with 16-fold variations in the HO/CA3 staining ratio in the presence and absence of Na-citrate are included in the measurements shown here. The variation in the relative peak position of the majority of human chromosomes is less than the channel width of the channel analyzer used (64x64 channel matrix). Only the variation in the relative peak position of the Y chromosome and of chromosome 13 exceeds this value.

DISCUSSION

This chapter describes some of the characteristics of the binding of Hoechst 33258, chromomycin A3 and propidium iodide to DNA in thymocyte nuclei and isolated chromosomes. The extent to which the binding kinetics

and the spectral interaction of these dyes influence bivariate flow karyotypes was determined. Since not all aspects of staining can be easily investigated on chromosomes in suspension, many of these studies were performed on thymocyte nuclei. At low dye concentrations or under non-equilibrium conditions, the fluorescence intensity distributions of chromosomes are too broad to allow recognition of individual chromosome peaks. A suspension of thymocyte nuclei can be prepared using the same procedure and buffer as are used to obtain chromosomes from mitotic cells. In situations where they could be compared directly, the staining properties of nuclei and chromosomes were similar. The amount of HO fluorescence quenched by CA3 binding and the effects of Na-citrate addition on dye fluorescence intensity are identical. It seems justified to conclude that findings obtained using thymocyte nuclei isolated in this way hold for chromosomes in suspension as well.

Addition of Na-citrate improves the chromosome fluorescence distributions obtained with a number of DNA dyes (chapter 7). Na-citrate changes the absolute fluorescence intensity of the dyes (table 8.1) and reduces the coefficient of variation of the fluorescence distributions of chromosomes and nuclei, but it does not affect the relative position of the chromosomes in a bivariate flow karyotype (figure 8.8).

The kinetics of the binding of DNA stains to unfixed nuclei in suspension were determined. HO staining is rapid and is completed in 5 min. PI staining is equilibrated after approximately 20-30 min. The binding of CA3 proceeds much more slowly. It may take several hours before the dye is in full equilibrium with chromatin. It is therefore important to stain chromosomes some time before flow karyotypes are measured. These findings concur with those reported by Darzynkiewicz and coworkers (1984) for the staining of fixed cells.

The binding kinetics of HO to nuclei already in equilibrium with CA3 are the same as the binding kinetics of HO to unstained nuclei (compare figures 8.4 and 8.6). This is also the case for CA3 staining of HO- or unstained nuclei. The change in the fluorescence of unstained nuclei, stained simultaneously with HO and CA3, can also be described by the kinetics of the individual dyes. These observations suggest that, at the concentrations tested, the two dyes do not compete for the same binding sites and interact primarily through resonance energy transfer. This is in agreement with previously reported observations on fixed cells (Langlois and Jensen, 1979) and unfixed chromosomes in suspension (Langlois et al., 1980).

The quenching of HO fluorescence by CA3 is proportional to the amount of CA3 bound to chromatin (figure 8.5). The measurements shown in table 8.2 have not been corrected for the spectral responses of the detectors

used. However, they give an indication of the magnitude of energy transfer and subsequent CA3 fluorescence of doubly-stained nuclei. The results shown here that, at the dye concentrations used for bivariate staining, approximately 65% of HO fluorescence is transferred to CA3. In the presence of Na-citrate, this value is 47%. A considerable amount of this transferred energy is reemitted as CA3 fluorescence. Approximately 33% of the detected fluorescence after HO excitation has the spectrum of CA3. In Na-citrate this value is approximately 13%. The differences in HO quenching and the contribution of CA3 to the measured fluorescence in the presence of Na-citrate are consistent with the reduction in CA3 binding. However, an increase in the length of chromosomes (Lucas et al., unpublished observations) in the presence of Na-citrate, may indicate that a decondensation of chromatin may contribute to the decreased amount of energy transfer in Na-citrate.

The magnitude of energy transfer from HO to CA3 observed here is similar to that reported previously. Langlois and Jensen (1979) reported energy transfer efficiency of HO to CA3 in single fixed cells of 79%. A transfer efficiency of 40% has been reported in chromosomes prepared in hexylene glycol (Langlois et al., 1980). Differences in these values may be attributed to differences in the chromatin organization in different isolation buffers, the staining concentrations used, and the spectral sensitivity of the collection optics used.

The measurements reported here demonstrate significant spectral interactions between HO and CA3. Considerable variation among human chromosomes in the amount of energy transferred from HO to CA3 exists. Figure 8.7 shows that the human chromosomes can be distinguished by measuring the relative amount of CA3 fluorescence after HO excitation in combination with the total fluorescence after HO excitation (right panel). The same chromosome groups are resolved in this distibution as are resolved in the conventional bivariate fluorescence distribution. This demonstrates that it is possible to measure bivariate flow karyotypes with UV excitation only. Although the resolution of this type of bivariate distribution is somewhat lower than that obtained with two lasers, this approach may be advantageous if the costs of a dual laser flow cytometer are prohibitive or if a conventional light source is used.

The changes observed in the relative peak positions of human chromosomes when the absolute and relative concentrations of HO and CA3 are varied over a wide range, are less than or equal to one channel width. Only chromosomes 13 and Y show detectable variation with staining conditions. These observations indicate that relative peak position in a conventional bivariate flow karyotype ((HOfl:HOex+CAfl:HOex) versus CAfl:CAex) is primarily the ratio of HO to CA3 bound to each chromosome,

and that energy transfer from HO to CA3 has only a secondary effect, if any, on relative peak position. In theory, shifts in the relative peak position of chromosomes along the HO axis could be expected with changes in staining conditions, if chromosomes, with the same HO/CA3 fluorescence ratio, differ in resonance transfer efficiency. Although the movement of chromosome 13 is indeed along the HO axis, its position changes less than 2-channel widths (3%) over a 16-fold concentration change. The change in the Y chromosome with a change in dye concentration is in CA3 fluorescence, and presumably does not reflect a change in the energy transfer efficiency.

The observations reported here suggest that, if variation in relative peak position occurs with changes in staining conditions, these variations are too small to be to be detected with the present generation of flow karyotype machines. For practical purposes, the staining conditions can be constrained so that little day to day variability will be encountered for a given cell source. This confirms the findings of Langlois et al. (1982) with human chromosomes isolated with a different procedure. Within these staining ranges, the relative peak position along the axis of HO fluorescence in a conventional flow karyotype is determined primarily by the amount of HO bound to the chromosome. It can be concluded that the resonance transfer efficiency and the average distances between HO and CA3 on most human chromosomes is the same. However, with the development of more sensitive machines, one should be prepared for variations in the relative peak positions of chromosomes, particularly of chromosomes 13 and Y, if staining conditions cannot be controlled.

Chapter 9

BIVARIATE FLOW CYTOMETRIC ANALYSIS OF CHROMOSOMES FROM SHORT-TERM AMNIOTIC CELL CULTURES

SUMMARY

Chromosomes from primary amniotic cell cultures were analyzed using dual beam flow cytometric analysis. Cultures were received three weeks after amniocentesis and were contact-inhibited. Trypsinizing, diluting and reculturing the cells for 48 h increased the number of dividing cells obtained from these cultures. Chromosomes were isolated from 10^3 to 10^4 mitotic cells in the 2.5 ml collected from each 25 cm^2 culture flask. High resolution bivariate fluorescence distributions suitable for quantitative karyotype analysis were obtained from 12 out of 14 of these chromosome suspensions after staining with Hoechst 33258 and chromomycin A3. Fetal sex can be determined from the flow karyotypes. The consequences of this study for the prenatal detection of Down's syndrome using flow cytometry are discussed.

INTRODUCTION

Amniocentesis and prenatal diagnosis form a widely accepted means of detecting genetic and metabolic defects and the sex of an unborn child (Fuchs, 1980). An important aspect of prenatal diagnosis is the analysis of the chromosomes, or the karyotype, of fetal cells. Routinely, amniotic fluid containing fetal cells is tapped at 16 weeks gestation (figure 9.1). The cells are cultured for approximately two weeks to increase the number of dividing cells. Mitotic cells are harvested and fixed to slides, and the chromosomes are banded for identification. When the chromosomes are analyzed microscopically, abnormalities such as trisomies, translocations, and large deletions can be detected. The detection of the Y chromosome is important for the early diagnosis of sex-linked inherited disorders. Diagnosis of chromosomal aberrations can usually be made before the 20th week of pregnancy (Galjaard, 1982).

It has been estimated that only 10-25% of those parents who would benefit by prenatal diagnosis have access to the technique (Galjaard, 1982). The technique is limited by the time needed to culture enough cells to obtain reliable chromosome spreads representative of the fetal cell

population and the time needed to analyze the chromosomes microscopically. Recognizing and classifying banded chromosomes is a highly specialized skill.

Automation of prenatal diagnosis by analyzing fetal chromosomes using flow cytometry could improve this situation. Samples can be processed very rapidly. Thousands of chromosomes can be analyzed in seconds. Flow cytometry is objective, and chromosomal properties can be expressed quantitatively rather than qualitatively. Previous results indicate that bivariate flow karyotypes can resolve approximately 20 chromosome groups in the human genome (Gray et al., 1979b; chapter 7).

The application of flow cytometry to amniotic cell research has been prevented by the lack of a procedure for the reliable isolation of chromosomes from fetal cell cultures. Several chromosome isolation methods require large numbers of mitotic cells ($5x10^6$ or more) for optimal resolution of chromosome groups in a flow karyotype (Gray et al., 1979b). Because past work has focused on the analysis of chromosomes from fibroblast cell lines (Gray et al., 1975a; 1979b, chapter 7) and peripheral lymphocyte cultures (Yu et al., 1981; Young et al., 1981; Matsson and Rydberg, 1981, chapter 7), this has been an inconvenience, but not a problem. In the measurement of the flow karyotype of chromosomes from amniotic cultures, this is a severe limitation.

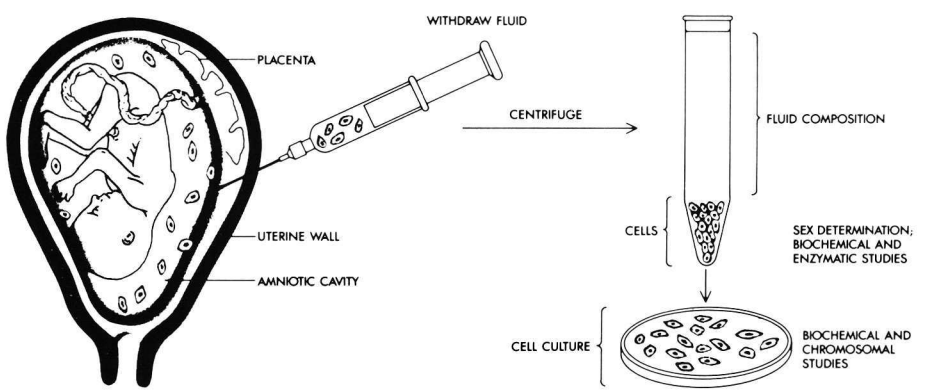


Figure 9.1. Amniocentesis. A sample of amniotic fluid (mostly fetal urine and other secretions) is taken by inserting a needle into the amniotic cavity around the 16th week of gestation. The fetal cells are separated from the fluid by centrifugation. The cells are then cultured so that a number of biochemical, enzymatic and chromosomal analyses can be made (reprinted with permission from Alberts et al., 1983).

The successful flow karyotyping of chromosomes prepared from short-term amniotic cultures is reported in this chapter. Flow karyotypes revealing quantitative information about the chromosomes in fetal cells can now be obtained at the same time after amniocentesis as conventional karyotyping.

MATERIALS AND METHODS

Amniotic cell cultures were received in 25 cm^2 flasks approximately 3 wk after amniocentesis had been performed (a gift from Dr. M.S. Golbus, University of California, San Francisco). The cultures were trypsinized by removing the growth medium and adding 2.5 ml Puck's Saline A solution containing 540 μM EDTA and 0.1% trypsin (Gibco, Grand Island, NY). This was removed after 30 s. When the cells loosened from the plate (usually after 3-5 min at room temperature), 5 ml alpha-MEM medium containing 15% fetal calf serum (FCS) was added. The cell suspension was pipetted vigorously and replated at various dilutions in new 25 cm^2 culture flasks containing 5 ml alpha-MEM medium plus 15% FCS. The cells were cultured at 37°C with 5% CO_2. The cultures were blocked in mitosis after 48 h in culture (at a cell confluency of approximately 20%) as described in chapter 7, with the exception that only 2.5 ml colchicine solution was added to each flask. The mitotic cells were harvested after 10-12 h at 37°C by shake-off. The procedure for chromosome isolation and the contents of the swelling buffer are as described (chapter 6), with the following modifications to scale down the volumes used. The medium collected from each culture flask (2.5 ml) and containing approximately 10^3 to 10^4 cells was resuspended after centrifugation in 0.5 ml hypotonic swelling buffer. The cells were disrupted with a 1-ml syringe after the addition of 50 μl 2.5% Triton X-100 solution. After the 30 min incubation at 37°C, the chromosomes were stained with 2.7 μM Hoechst 33258 (HO) and 27 μM chromomycin A3 (CA3) and placed on ice. Three hours after staining and 1 h before flow cytometric analysis, 50 μl sodium citrate (Na-citrate) were added to 10 mM final concentration. The chromosome suspensions were analyzed using the Livermore dual beam cytometer (Dean and Pinkel, 1978) with 1 W of laser power for UV excitation, and 300 mW output at 458 nm. Although the concentration of chromosomes varied in the different preparations, chromosomes were analyzed at a rate of approximately 200 events/s.

RESULTS AND DISCUSSION

The way in which amniotic cells are cultured and the time when the chromosomes are isolated is important in obtaining high resolution flow karyotypes of these cells. The primary amniotic cultures used in this study were surplus, three week-old, contact-inhibited cultures when they were received from a fetal karyotyping laboratory. Cultures blocked as they were received (without trypsinization and dispersal) (6 cultures) and cultures trypsinized and dispersed on the original plates (12 cultures) gave unsatisfactory chromosome distributions. However, by increasing the number of dividing cells through trypsinization, replating at a lower dilution, and culturing for 48 h, the number of cultures from which good chromosome suspensions could be isolated was increased. Twenty cultures (in 25 cm^2) flasks were trypsinized and split at various dilutions into new 25 cm^2 culture flasks. Six cultures did not show recovery after trypsinization. The number of dividing cells varied among the remaining 14 cultures; cell density at the time of colchicine addition was less than 30%

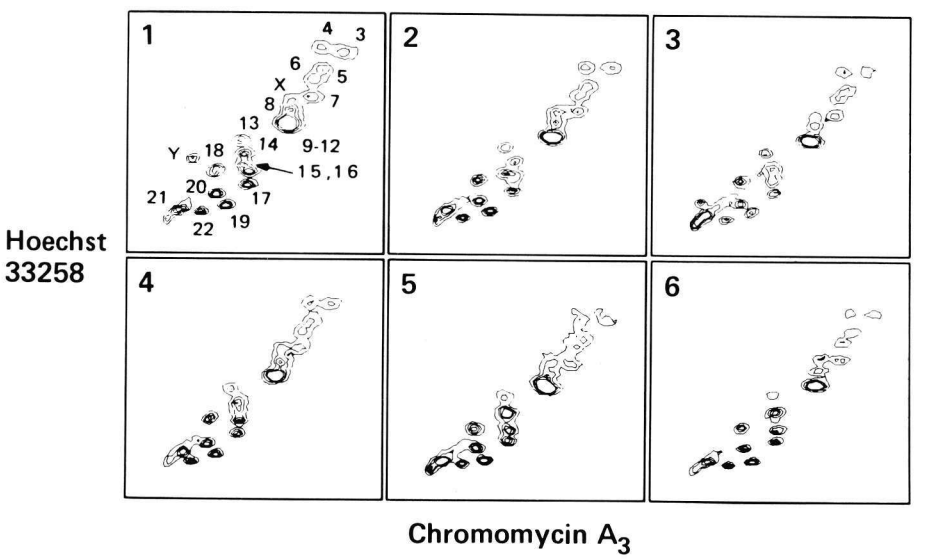


Figure 9.2. Bivariate fluorescence distributions of chromosomes prepared from six amniotic cultures. Numerical labels of the peaks in panel 1 indicate the chromosomes responsible for each peak. In these distributions, the amplifier gains were adjusted such that all chromosomes except numbers 1 and 2 are displayed. The number of days elapsing between amniocentesis and the measurement of the flow karyotype in each panel is as follows: panels 1, 4, 5 and 6: 24 days; panel 2: 27 days; panel 3: 21 days.

confluency. In 12 of the 14 cases, the 1:2 split was chosen to be used for chromosome isolation; two cases, the 1:6 split was chosen.

Chromosomes were isolated from the 2.5 ml of cells obtained from each of these 14 cultures after a 10 h colchicine block. Excellent chromosome preparations, for which high resolution bivariate distributions could be measured in a dual beam cytometer, were obtained from 12 out of the 14 cultures, despite the low numbers of cells collected.

The bivariate distribution of the chromosomes from six representative amniotic cultures are shown in figure 9.2. The chromosomes responsible for the peaks are indicated by the numbers near each peak in the first panel. The larger human chromosomes (numbers 1 and 2) are not displayed in these distributions. Chromosome assignment is based on previous human chromosome analysis using fibroblast cultures (Gray et al., 1979b; Carrano et al., 1979a; Yu et al., 1981). The fetuses whose chromosomes are analyzed in panels 1 and 3 are males; the others are females. Note the heterogeneity in the Y chromosome and chromosomes 22 (panel 4) and 13-17 (panel 2).

These fluorescence distributions are suitable for quantitative karyotypic analysis. The resolution obtained in the flow karyotypes of amniotic cells can be compared to that obtained from the human fibroblast cell line shown in chapter 7. Each chromosome, except those of the 9-12 group, is resolved. Small fluorescent particle debris, which can interfere with the resolution of the smaller chromosomes (especially the 21), was low in these preparations. Chromosome clumping, which results in a continuum of particles underlying the peaks, was also low.

The average number of days elapsing between amniocentesis and the measurement of a bivariate fluorescence distributions was 24 days for the 14 cultures. This is within the same time span necessary for conventional karyotyping. It is likely that cultures obtained from the clinic earlier than three weeks and exhibiting less contact inhibition will respond better to trypsinization and provide consistently higher numbers of dividing cells. Optimization of the trypsinization and culture conditions should allow further reduction in the time elapsing between amniocentesis and flow karyotypic analysis.

Experiments are now in progress to prepare and analyze the chromosomes isolated from a large number of amniotic cultures using the procedures described in this paper (Gray, personal communication). In this study, the quantitative flow karyotype analysis of each culture is compared to the results from conventional banding analysis in an effort to demonstrate the detection capabilities of flow karyotyping. With flow cytometry, one has the advantage that a large number of chromosomes can be examined rapidly, quantitatively, and objectively. Several cases of trisomy of chromosome

21, Down's syndrome, have been confirmed with this technique (Gray, personal communication).

The results described in this paper open the possibility that the flow karyotype analysis of fetal cells can be performed on a routine basis. This could conceivably increase the number of people who are able to benefit from prenatal diagnosis, in addition to giving a quantitative basis to the study of genetic abnormalities. The method should prove particularly useful for the rapid determination of fetal sex in cases where sex-linked disorders are suspected.

Chapter 10

MITOTIC CELL ENRICHMENT BY EQUILIBRIUM DENSITY SEPARATION

SUMMARY

The enrichment of mitotic cells from bone marrow of normal rats is described. Mitotic cells are less dense and can be separated from the majority of interphase cells in a density gradient. Cell suspensions containing 50% mitotic cells, on average, can be obtained in this way. Density gradient separation has no effect on the DNA distributions measured in a flow cytometer of chromosome suspensions prepared from in vitro rat rhabdomyosarcoma cells.

INTRODUCTION

Human leukemias are associated with specific chromosomal lesions (Rowley et al., 1966; Rowley, 1973; Yunis, 1983; Zech et al., 1976). Flow karyotyping offers the possibility to quantify the amount of DNA involved in these lesions and the frequency of aberrant cells, if suitable chromosome suspensions can be prepared from bone marrow cells. Flow sorted fractions of aberrant chromosomes from leukemic cells could also be used for gene mapping studies or for the construction of recombinant DNA libraries.

Bone marrow cells are not an ideal source of cells for the preparation of chromosome suspensions. Only a small fraction of cells can be collected in mitosis. Interphase nuclei are more fragile than the interphase cells from fibroblast cultures. These factors may result in small fluorescent particle debris, reducing the ability to discern small new peaks in a flow karyotype, indicative of aberrant chromosomes. Methods for the enrichment of mitotic cells in bone marrow suspensions are required.

In this chapter, density gradient separation is shown to be effective in increasing the concentration of mitotic cells in suspensions of rat bone marrow cells. This treatment has little effect on chromosome resolution of the flow karyotype of rat rhabdomyosarcoma culture cells.

MATERIALS AND METHODS

Animals and cell cultures

Bone marrow suspensions were prepared from normal male Brown Norway (BN) rats (bred in Rijswijk) at approximately 17 weeks of age. Rat rhabdomyosarcoma (RS) cells (a gift from Dr. G. Barendsen, Rijswijk) were cultured in alpha-MEM (Gibco) medium supplemented with 10% fetal calf serum in 75 cm^2 culture flasks.

Mitotic arrest and cell collection

Rats were injected i.v. with 600 μg/kg body weight vincristine (Eli Lilly, Indianapolis, IN) 16 h before bone marrow cells were collected. The animals were supplied food and water ad libitum. Bone marrow cell suspensions were made in Hanks balanced salt solution buffered with Hepes (HH) containing 0.1 μg/ml vincristine. These cells were separated according to their density as described below.

To collect RS cells in mitosis, the cells were culture in 10 ml fresh growth medium containing 0.1 μg/ml colcemid for 16 h. Mitotic cells were collected by shake-off. Alternatively, RS cells were treated with colcemid as above, but trypsinized to collect both interphase and mitotic cells. These RS cell suspensions were used in the density separations.

Discontinuous density gradients

Cell suspensions were separated on Nycodenz (Nyegaard and Co., Oslo) density gradients prepared at pH 6.5, with an osmolarity of 283 mosm, and containing 0.1 μg/ml vincristine. The densities indicated in the results section are the densities of Nycodenz at 20°C as measured on a densitometer (Mettler/Paar DMA 40). Cells were centrifuged 10 min at 100g and resuspended in 2 ml dense Nycodenz at a cell concentration of $5x10^7$-10^8 cells/ml. After careful mixing, 2 ml light Nycodenz was gently layered on top. The gradients were centrifuged 350g for 30 min at 20°C. The interface between the two layers was collected by careful pipetting.

Mitotic index determination

The recovery of nucleated cells was determined by counting the number of cells in the original suspension and in the interface band after Turck's staining. Mitotic cell preparations were made to determine the mitotic index of the original cells and those collected at the gradient interface. Cells were diluted in HH and centrifuged 10 min at 100g. The cell pellet was resuspended for 10 min in 75 mM KCl containing 0.1 μg/ml vincristine at 37°C. The swollen cells were centrifuged at 100g for 10 min at 4°C and were resuspended slowly in fresh fixative while vortexing (3 volume parts

methanol: 1 volume part glacial acetic acid). The fixed cells were centrifuged 250g for 10 min at 4°C. The fixation steps were repeated twice. The concentrated cell pellet was dropped onto microscope slides and stained with 3% Giemsa in phosphate buffer, pH 6.8. The frequency of mitotic cells was determined microscopically.

Preparation of chromosome suspensions

RS cells collected at the interface of the discontinuous density gradient, or mitotic cells obtained directly from RS cultures, were diluted in HH and centrifuged at 100g for 10 min at 4°C. The chromosomes were isolated as described in table 6.1 with the exception that the KCl concentration in the buffer was reduced to 25 mM to isolate chromosomes from the cells obtained from the gradients.

Flow cytometric analysis

Chromosome suspensions were analyzed in a FACS flow cytometer (Becton-Dickinson, Sunnyvale, CA) in Rijswijk after propidium iodide (PI) (20 μg/ml) staining. The chromosomes were illuminated by 600 mW light at 488 nm from a Coherent Innova 90 laser (Palo Alto, CA), and propidium iodide fluorescence was measured through a KV 590 emission filter (Schott, Mainz).

RESULTS

The mitotic index of bone marrow cells of normal rats treated with vincristine for 16 h is approximately 11% (average of 7 experiments, range 9-13%).

To determine if the number of mitotic cells in a suspension of rat bone marrow cells could be enriched on the basis of their density, a series of discontinuous density gradients were prepared using 0.005 g/cm^3 density intervals. Figure 10.1 shows the averaged results of two experiments. The suspension with the highest frequency of mitotic cells could be collected at densities between 1.055 and 1.070 g/cm^3. The recovery of mitotic cells also peaked at these densities. The majority of the nucleated cells were collected at higher densities.

The highest frequency of mitotic cells was also found at densities between 1.055 and 1.070 g/cm^3 for rat rhabdomyosarcoma culture cells treated with colchicine and trypsinized to collect both interphase and mitotic cells (data not shown).

Discontinuous density gradients can be used to increase the mitotic index of suspensions of rat bone marrow cells. Table 10.1 shows the results of a series of 11 experiments in which bone marrow cells of normal

rats, treated in vivo with vincristine, were separated in discontinuous density gradients. Suspensions of cells containing, on the average, 51% mitotic cells can be obtained routinely. This is an average enrichment of 4.7 times. This enrichment is balanced, however, by the recovery of only 10% of the original mitotic cells. The recovery of nucleated cells at the interfaces of these gradients averages 2%.

Chromosomes can be isolated from the mitotic cells of rat rhabdomyosarcoma culture cells collected from discontinuous density gradients. Chromosomes were isolated as described in chapter 6 from the cells collected from the interface between density layers of 1.055 and 1.070 g/cm^3. The fluorescence distribution of the chromosomes after DNA staining (figure 10.2A) is approximately identical to the distribution of chromosomes isolated from mitotic cells shaken directly from the cultures (figure 10.2B).

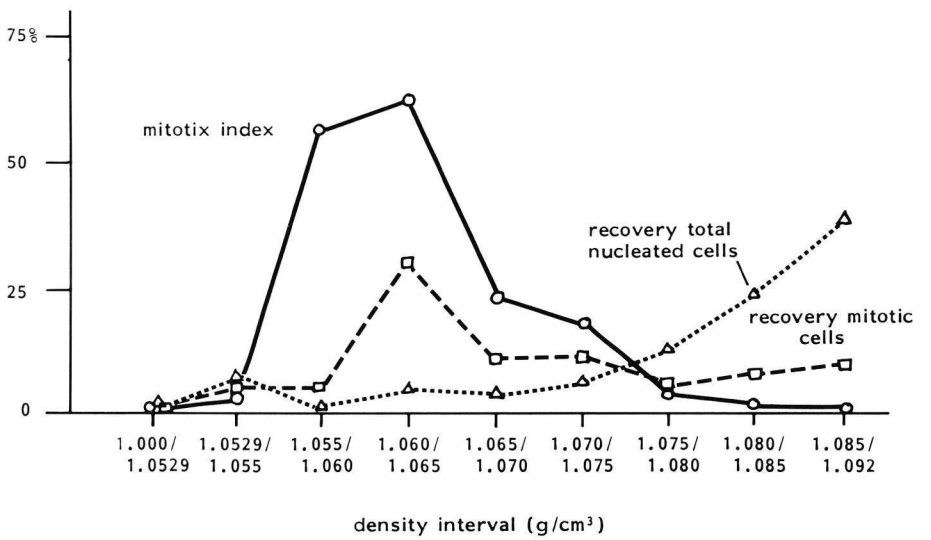


Figure 10.1. Enrichment of mitotic cells in bone marrow cell suspensions using discontinuous density gradients. Nine different gradients, as indicated along the X-axis, were prepared. $5x10^7$ bone marrow cells from vincristine-treated rats were centrifuged in each of these gradients. The recovery of nucleated cells (△), the recovery of mitotic cells (□) and the mitotic index (○) of collected fractions are plotted along the ordinate. These values represent the average of two experiments. The mitotic index of the original suspension averaged 10%.

Table 10.1

MITOTIC ENRICHMENT BY DISCONTINUOUS DENSITY GRADIENT SEPARATION OF NORMAL RAT BONE MARROW CELLS

expt.	mitotic cell index of original cell suspension (%)	density interval in gradient (g/cm^3)	mitotic index of collected cells (%)	enrich-ment factor	recovery of nucleated cells (%)	recovery of mitotic cells (%)
I	10	1.060/1.065	62	6.2	9	56
II	10	1.060/1.065	39	3.6	1	3
		1.055/1.070	37	3.4	3	9
III	9	1.060/1.065	62	6.9	1	4
IV	9	1.055/1.065	50	5.7	1	5
V	11	1.055/1.065	40	3.6	3	10
VI	12	1.055/1.065a	68	5.7	2	10
		" b	57	4.8	1	14
		" c	52	4.7	1	10
		" d	54	4.5	1	9
VII	13	1.055/1.065	39	3.0	1	2
Average =	11		51	4.7	2	12

*a-d: density gradients prepared from same bone marrow suspension using cell concentrations of 10^8, 5×10^7, 10^7 and 5×10^6, respectively.

DISCUSSION

It is possible to enrich for mitotic cells in rat bone marrow cell suspensions by capitalizing on the density differences between mitotic and interphase cells. Suspensions containing 5-6 times more mitotic cells than the original suspensions can be obtained. This treatment does not, in itself, affect the isolation of chromosomes, as far as could be determined in the flow cytometer used. Flow karyotypes measured for rat rhabdomyosarcoma cells after density separation were identical to those measured for mitotic cells obtained by "shake-off".

In cases where the mitotic index, and not the recovery of cells, is important, the density separation procedure described here may be useful. Mitotic indices of approximately 50% could be obtained routinely at the interface between two solutions of different density. This method carries the risk that selective loss of certain mitotic cell populations may occur. A selective loss is undesirable in studies designed to detect and quantify the frequency of aberrant cells in a bone marrow suspension.

Preliminary results indicate that chromosome suspensions prepared from bone marrow cells contain high levels of small fluorescent particle debris,

even after mitotic cell enrichment. Experiments are in progress to pinpoint the source of this debris. The interphase cells and mitotic chromosomes from bone marrow may be significantly more fragile than those from other tissues and may be fragmented during the shearing step. Bone marrow suspensions from animals treated with vincristine for 16 h may also contain a high number of dead cells, which may contribute to the debris continuum. Treatment of cell suspensions with DNase before chromosome isolation (Kooi et al., 1983) may alleviate this problem.

Improvements in the procedure for isolating chromosomes from bone marrow suspensions enriched for mitotic cells, as well as in the flow cytometers used for their analysis, should allow the detection and quantification of abnormal chromosomes in these tissues.

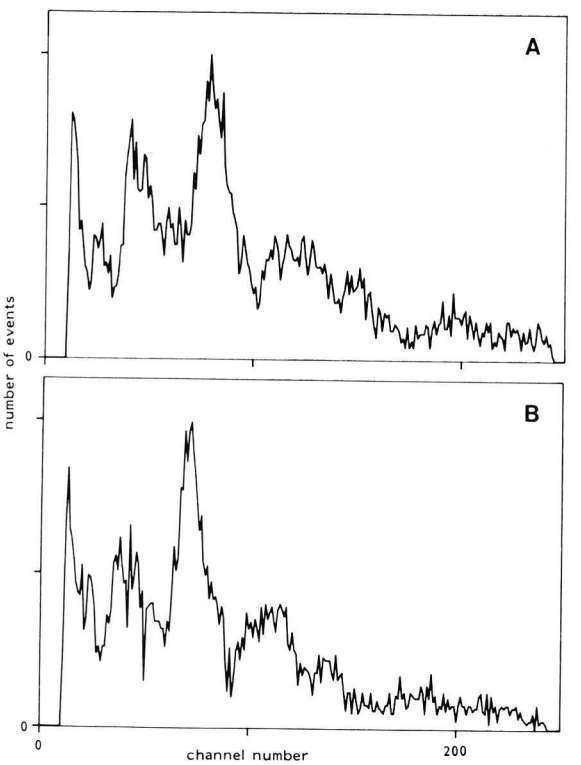


Figure 10.2. (A) Univariate fluorescence distributions of chromosomes isolated from rat rhabdomyosarcoma in vitro culture cells collected with trypsin after colchicine treatment and enriched for mitotic cells by discontinuous density gradient separation. (B) Univariate fluorescence distribution of chromosomes isolated from the mitotic cells collected by shake-off from in vitro cultures of rat rhabdomyosarcoma cells; No density gradient separation was employed before chromosome isolation. The chromosome suspensions in both (A) and (B) were analyzed in a FACS flow cytometer after PI staining.

SECTION III

STRUCTURAL ORGANIZATION OF CHROMOSOMES

Introduction

In this section, studies on the structure of chromosomes prepared by the Hepes/$MgSO_4$ method are presented. The DNA and the protein composition of chromosomes appear to be sufficiently preserved during isolation that the chromosomes can serve in further morphological and biochemical analyses. Methods are explored for labeling chromosomal proteins with fluorescent antibodies. Further, a fixation technique for preserving the structure of chromosomes is introduced so that chromosomes in suspension can be made suitable for light microscopical examination.

The emphasis in the preceding chapters has been placed on the use of DNA-specific stains as molecular probes of chromosome content. Chromatin consists, however, of both protein and DNA. It is through the interaction of protein and DNA that chromatin obtains its compact structure. There is also some evidence that the organization of DNA and proteins in regions of the genome being actively transcribed differs from that in quiescent regions (see review, Reudelhuber, 1984). Figure III.1 depicts the association of the histone proteins and the linear DNA helix molecule in chromatin. The DNA helix is wrapped around a group of eight histone molecules to form a unit called the nucleosome. The string of nucleosomes is coiled to form a thicker fiber, which in turn is tightly wound to form the chromosome. This conformation has been studied using a variety of techniques, including electron microscopy, circular dichroism spectroscopy, and differential enzyme digestion after cross-linking. Antibodies have also been used to probe nucleosome structure in chromosomes fixed to slides. It is this structure that also is thought to underly the banding patterns that can be made visible in chromosomes after certain staining procedures (Van Duijn et al., 1985).

If the DNA helix or the structural proteins in chromatin are damaged during chromosome isolation, one can assume that the chromosome morphology, the molecular weight of the DNA and the extractable proteins will be altered. In these chapters, results are presented which indicate that chromosome morphology is preserved. In chapter 11, the fragment sizes of the DNA extracted from chromosomes isolated using several different protocols are compared. The size of the DNA fragments that can be

extracted from chromosomes isolated using the Hepes/$MgSO_4$ procedure, described in section II, is similar to that of fragments extracted from chromosomes isolated using other procedures. In chapter 12, the histone proteins of unstained, stained and sorted chromosomes are analyzed and shown to be unaffected by the chromosome isolation procedure.

Evidence is given in chapter 12 to show that chromosomes can be labeled in indirect immunofluorescence procedures so that specific antibody fluorescence as well as the DNA content of individual chromosomes can be analyzed in a flow cytometer. One of the structural proteins in chromosomes, histone 2B, is quantitatively studied using a monoclonal antibody specific for this protein. Indirect immunofluorescent labeling procedures (figure III.2) entail incubation of the chromosomes in the presence of anti-histone 2B antibody. After the antibody has had sufficient time to bind to accessible molecules of histone 2B in the chromosomes, the chromosomes must be washed to remove unbound antibody molecules. The chromosomes are then incubated in anti-immunoglobulin antibody conjugated to fluorescent molecules, such as fluorescein. This antibody specifically binds to the anti-histone 2B molecules. The chromosomes are washed again to remove free anti-immunoglobulin molecules. The entire procedure must take place with minimal chromosome loss and with minimal chromosome aggregation. To demonstrate the effect of

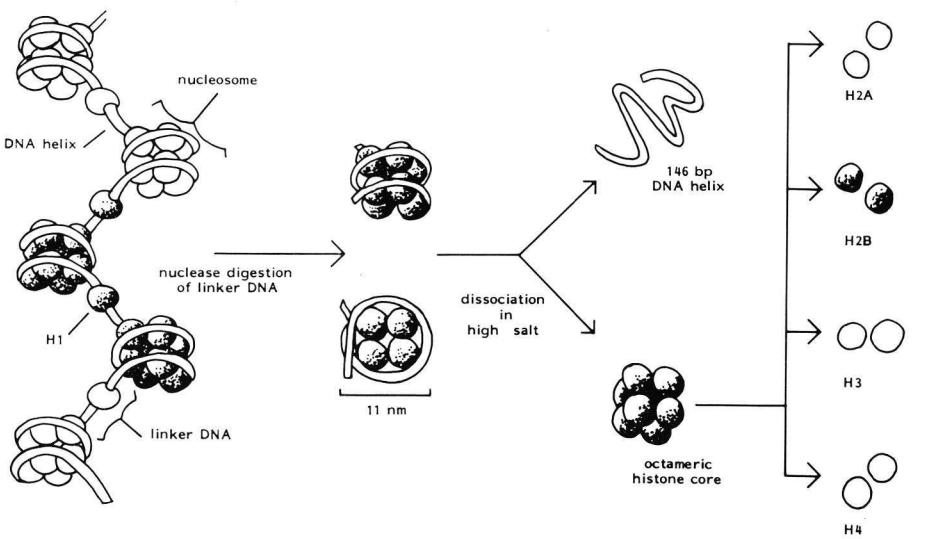


Figure III.1. Schematic representation of the association of histones and the DNA helix in chromatin (redrawn from Alberts et al., 1983).

immunofluorescent labeling procedures on chromosome suspensions, the DNA in the chromosomes was stained. Chapter 12 shows that the DNA distributions of chromosomes are not significantly affected by immunofluorescent labeling procedures.

The fluorescence intensity of chromosomes labeled with anti-histone 2B antibody in this procedure was quantified in a flow cytometer. The results indicate that the intensity of fluorescence from bound antibody is proportional to the DNA content of each chromosome. This is consistent with the stoichiometry of the association of DNA and histones in chromatin. Differences were observed, however, in the amount of the anti-histone 2B antibody that could be bound to the chromosomes from different mammalian species. Furthermore, intercalating DNA stains also affect the binding of this antibody to chromosomes in suspension. These observations indicate that quantification of the binding of antibodies to chromatin components with flow cytometry is a new and alternative approach to the study of the structural organization of chromosomes.

Another aspect of chromosome structure is revealed in the banding patterns obtained in chromosomes on slides after certain staining procedures. Although their origin is not well understood, some banding patterns are thought to reflect the organizational structure of the DNA and protein complex in chromatin (Van Duijn et al., 1985). The results in

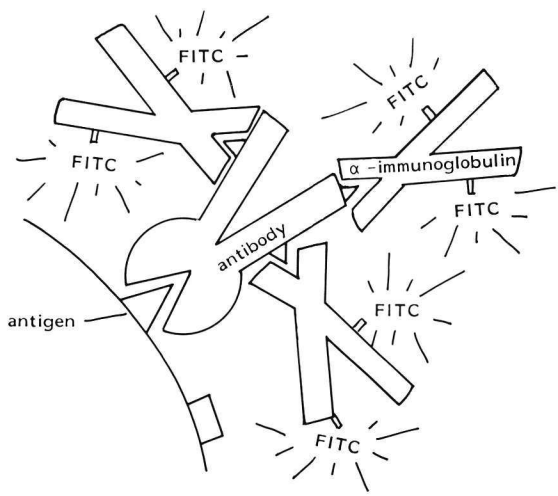


Figure III.2. Schematic representation of the fluorescent labeling of an antigen using an indirect immunofluorescence technique. An antibody that specifically recognizes the antigen binds to it. An antibody that binds specifically to immunoglobulin molecules forms the second layer. The anti-immunoglobulin is conjugated to fluorescent FITC molecules. In this way, several fluorescent molecules mark the location of each antigen molecule.

chapter 11 show that the structures necessary for the production of bands are preserved in isolated chromosomes. Isolated chromosomes can be stabilized, fixed to slides and stained. The chromosomes show normal morphology and banding patterns sufficient for their identification.

This section shows that flow cytometry can be used to study the structural organization and protein content, as well as the DNA content, of single chromosomes. The observation that chromosomes can be labeled in suspension with fluorescent antibodies makes the wealth of these reagents available for the flow cytometric study of chromosome structure and content.

The studies in this section also bring to light several advantages of the isolation method described in section II. If sorting is to be used to purify chromosomes so that the DNA they contain can be cloned, the molecular weight of the DNA that can be extracted from isolated chromosomes should be high. In this way the full insert capacity of cloning vectors can be utilized. This reduces the numbers of clones that may be needed to cover a complete gene sequence and simplifies "walking" along the chromosome. In addition, it is often desirable to identify sorted chromosomes by banding. This is helpful in identifying the chromosomes responsible for peaks in a karyotype, or in checking the purity of sorted fractions.

Chromosome recovery from mitotic cells has been reported to be low (Gray, personal communication). This is a problem when chromosomes are to be sorted for the production of chromosome-specific genomic libraries. In chapter 11, the recovery of chromosomes from mitotic cells is investigated for a variety of mammalian species. Chromosome recovery is shown to range from 23 to 78% using the Hepes/$MgSO_4$ protocol. This high recovery should help reduce the scale of cell cultures required for the purification of sufficient quantities of chromosomes.

Chapter 11

STUDIES ON ISOLATED CHROMOSOMES: BANDING, MOLECULAR WEIGHT OF DNA AND CHROMOSOME RECOVERY

SUMMARY

The size of the DNA fragments that can be extracted from chromosomes isolated using the Hepes/$MgSO_4$ procedure described in chapter 6 is larger than 50 kbase pairs. This is sufficiently large for most recombinant DNA cloning procedures. The size of the DNA fragments obtained from chromosomes isolated using other chromosome preparation procedures are similar. The recovery of chromosomes from mitotic cells using the Hepes/$MgSO_4$ method is high (25-75%). Recovery in this order was obtained from the mitotic cells from fibroblast cell cultures of several mammalian species. In addition, the majority of isolated chromosomes can be banded on slides for their identification and further study. The protocol for banding isolated chromosomes requires their stabilization with a protein cross-linker, dimethylsuberimidate. Bands can be produced in these chromosomes after a hot SSC/trypsin/Giemsa staining procedure.

INTRODUCTION

Flow cytometry and sorting can be used for the purification of chromosomes for gene mapping studies (Lebo et al., 1979; Collard et al., 1984) or for the construction of chromosome-specific recombinant DNA libraries (Davies et al., 1981). Important requirements in this application are high flow karyotype resolution, high chromosome recovery from the cell source being used, little or no damage to the DNA or proteins in isolated chromosomes, and the ability to band the chromosomes after sorting using conventional cytogenetic techniques.

In previous chapters, high resolution flow karyotypes have been shown for human chromosomes isolated from several cell types using the Hepes/$MgSO_4$ procedure (chapters 7,9). Variability in the flow karyotypes is low even when the staining conditions are varied (chapter 8). This chapter presents the results of experiments designed to assess the

yield, the molecular weight and the ability to band chromosomes isolated using this method.

The manner in which chromosomes are prepared has been shown to have an affect on the size of DNA fragments that can be isolated from them (Blumenthal et al., 1979). One major concern in the Hepes/$MgSO_4$ method is that nucleases, which require divalent cations for activity and may be present in the suspension, will digest chromosomal DNA, making the procedure unsuitable for recombinant cloning techniques. DNA fragments ranging from 20-50 kb have been isolated from these chromosomes (chapter 7). This suggests that, at least for some cell sources, the presence of Mg^{++} and endogenous DNase will have neglible effects on the molecular weight of DNA. The experiments described in this chapter were designed to compare the molecular weight of the DNA extracted from chromosomes isolated with the Hepes/$MgSO_4$ method with that from chromosomes isolated with o isolation procedures (polyamine: Sillar and Young, 1981; citric acid: Collard, 1980; hexylene glycol: Yu et al., 1981). The sizes of the extracted DNA fragments are compared using agarose gel electrophoresis (Maniatis et al., 1982).

Sorting and identifying the chromosomes responsible for peaks in the flow karyotype can be an aid to karyotyping (Carrano et al., 1979a, b), an aid in the identification and cytogenetical study of chromosomes producing new peaks in a flow karyotype (Bernheim et al., 1983), and a means to check the purity of sorted chromosomes (Carrano et al., 1979a, b). For these purposes, one would like to collect chromosomes, fix them to slides, and produce a banding pattern in the chromosomes by which they can be identified. The ease with which this can be done is related to the chromosome isolation procedure. Chromosomes isolated using the hexylene glycol method have been successfully banded using quinacrine staining after sorting (Carrano et al., 1979a, b; Gray et al., 1979b, Yu et al., 1981). Approximately 15% of the total number of sorted chromosomes, could be identified (Carrano et al., 1979b). Chromosomes prepared using a Tris/$MgCl_2$ method (Otto et al., 1980) could be banded with quinacrine after sorting if hexylene glycol was used as sheath fluid (Disteche et al., 1982). Chromosomes isolated using the polyamine buffer (Sillar and Young, 1981) have resisted attempts at banding (Sillar and Young, 1981; Buys et al., 1982, Davies et al., 1981, Bernheim et al., 1983), although recent reports suggest that these chromosomes can also be identified by quinacrine banding (Lebo et al., 1984; Fantes et al., 1983). The majority of chromosomes isolated using the PI method (Aten et al., 1980) can be banded, if they are fixed and stained with DAPI and actinomycin D (Buys et al., 1982). This chapter presents a procedure by which chromosomes isolated in using the Hepes/$MgSO_4$ procedure can be banded.

In the large scale purification of individual chromosome types for the construction of chromosome-specific recombinant libraries (Davies et al., 1981), one of the major limitations is the low recovery of chromosomes from mitotic cells. This low yield means that large numbers of cells must be cultured for the production of sufficient numbers of chromosomes (Gray, personal communication). For this reason, the recovery of chromosomes from the mitotic cells of a variety of mammalian species was investigated.

MATERIAL AND METHODS

Cell sources

The following cell cultures were used as a source of mitotic cells: 1) a human diploid fibroblast culture (MBL) , a gift from Dr. R.A. Baan, Medical Biological Laboratory, Rijswijk; 2) a Chinese hamster fibroblast cell culture (NBCH), a gift from DR. G. Barendsen, Rijswijk; 3) a mouse fibroblast culture derived from the NIH/3T3 cell line, a gift from Dr. V. Krump, Rijswijk; 4) a transformed human fibroblast cell line (HEP), a gift from Dr. H. Schellekens, Rijswijk; and 5) a rat rhabdomyosarcoma in vitro cell culture (RS), a gift from Dr. G. Barendsen. All cultures were maintained in exponential growth as described previously (chapter 6).

Chromosome isolation

Chromosomes were isolated from the mitotic cells collected by shake-off after colcemid (Sigma, 0.1 μg/ml) treatment of the cell cultures. The cell cultures were trypsinized and replated at a 1:2 or 1:3 dilution, approximately 24 h before colchicine addition. Cultures were incubated in colchicine for 6 h (Chinese hamster) or 16 h (human, rat and mouse). Chromosomes were isolated according to the procedure described in chapter 6. Where indicated, the chromosomes were stained with 2.7 μM Hoechst 33258 and 27 μM chromomycin A3 (both Sigma) and treated with 10 mM sodium citrate (Na-citrate).

Chromosomes were also isolated from Chinese hamster fibroblasts using the citric acid procedure (Collard et al., 1980), the polyamine procedure (Sillar and Young, 1981) and the hexylene glycol procedure (Yu et al., 1981) as described in these publications.

Dimethylsuberimidate (DMS) treatment

For some experiments, the chromosomes were stabilized with dimethylsuberimidate (DMS). For DMS treatment, K_2CO_3 and DMS were added to a suspension containing approximately 10^7 chromosomes/ ml from a 5 times concentrated stock solution mixed immediately before use. The final concentrations were 20 mM and 3 mM, respectively.

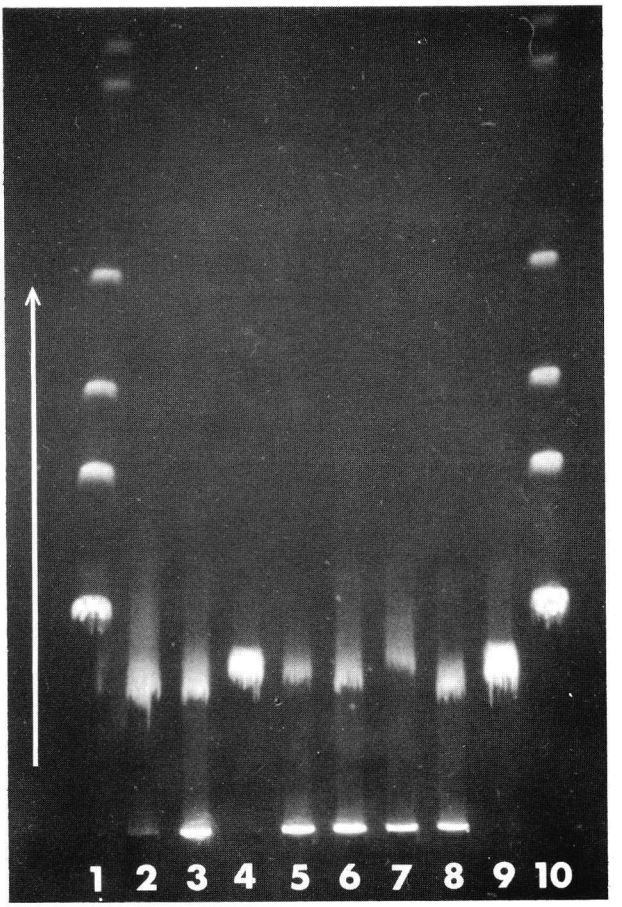


Figure 11.1. A representative agarose gel (0.3%) after electrophoresis showing the molecular weights of the DNA extracted from chromosomes isolated from Chinese hamster fibroblast cells according to several procedures. Lanes were filled at the bottom, and the fragments were electrophoresed towards the top. The lanes contain, from left to right:

1) Hind III restriction enzyme digest of bacteriophage lambda, sizes: 23.5, 9.7, 6.6, 4.5, 2.2 and 2.1 kbase pair (kb);
2) mouse DNA;
3) DNA isolated from chromosomes prepared using the Hepes/$MgSO_4$ procedure;
4) intact lambda bacteriophage, size: 50 kb;
5) DNA isolated from chromosomes prepared according to the polyamine procedure;
6) DNA isolated from chromosomes prepared according to the citric acid procedure;
7) DNA isolated from chromosomes prepared according to the hexylene glycol procedure;
8) DNA isolated from cells trypsinized from cultures and not exposed to any of the chromosome isolation buffers;
9) intact lambda bacteriophage as in lane 4;
10) Hind III digest of lambda bacteriophage, as in lane 1.

The resultant pH was 10. After 15 min at 25°C, the pH was adjusted to 8.0 by the addition of 50 µl 100 mM citric acid/ml.

Determination of cell and chromosome recovery

The number of cells obtained by shake-off and the number of cells recovered after resuspension of the cell pellet in isolation buffer were counted in a hemacytometer after Turck's staining. The mitotic index of both cell suspensions were determined. After the chromosomes were released into suspension, the number of chromosomes was determined using a hemacytometer and a fluorescence microscope (x40 oil objective) after staining with 2 µg/ml Hoechst 33258.

DNA isolation

DNA was extracted from Chinese hamster chromosomes isolated from approximately $5x10^6$ cells according to several procedures, or from Chinese hamster tissue culture cells harvested by trypsinization. The procedure for DNA extraction is that described by Maniatis et al. (1982) for the isolation of high molecular weight DNA.

Gel electrophoresis

The size of isolated DNA fragments was determined by electrophoresis in a 0.3% agarose gel containing 0.5 µg/ml ethidium bromide using Tris-borate buffer as described by Maniatis et al. (1982). The gels were subjected to 5 mamp (20-30 V) overnight. In some cases, isolated DNA was heated 5 min at 60°C before application to the slot. The gels were photographed using transmitted UV light with Polaroid positive/negative Land film type 55, for 30 min.

The size markers used were 1) intact bacteriophage lambda DNA (Bethesda Research Laboratory, Isenberg, W. Germany), approximately 50 kbase pairs (kb) in size; 2) HIND III restriction enzyme fragments of lambda DNA (New England Biolaps, Beverly, MA), approximately 23.5, 9.7, 6.6, 4.1, 2.2, and 2.1 kb in size; and 3) high molecular weight DNA extracted from mouse cells (a gift from A. Van den Ouweland, Nijmegen).

RESULTS

Molecular weight determination of the DNA isolated from chromosomes

Experiments were performed to determine and compare the size of the DNA fragments that could be extracted from Chinese hamster chromosomes isolated with the Hepes/$MgSO_4$ method and several other chromosome isolation procedures. Chromosomes were isolated using the Hepes/$MgSO_4$ method, the citric acid method, the polyamine buffer, and

the hexylene glycol procedure. DNA was isolated from these chromosomes, without sorting or other purification, using the techniques described by Maniatis et al. (1982) for preservation of high molecular weight DNA. The isolated DNA was subjected to electrophoresis in 0.3% agarose gels. A representative gel is shown in figure 11.1. The DNA fragments isolated from all chromosome suspensions except those prepared in the hexylene glycol buffer migrate slower than the 50 kb lambda molecular weight marker. The DNA fragments extracted from chromosomes isolated using the hexylene glycol method migrate aproximately with the lambda marker. The molecular weight weight of DNA isolated from culture cells, which were harvested by trypsinization and not exposed to chromosome isolation procedures, was also in this range.

Table 11.1

RECOVERY OF CHROMOSOMES ISOLATED FROM MITOTIC CELLS OF VARIOUS SPECIES USING THE $MgSO_4$ ISOLATION PROCEDURE

cell line	number of cells collected in mitotic shake-off/ml ($\times 10^4$)	mitotic index of cells at shake-off (%)	recovery of mitotic cells at swelling step (%)	recovery of chromosome with respect to original collected (%)*
Chinese hamster a	5.6	88	76	60
" b	9.0	85	72	78
" c	11	90	74	25
" d**	5.6	88	50	28
" d	5.6	88	27	56
Average values for Chinese hamster =		90	60	49
human HEP***	48	95	52	53
mouse NIH a	6.3	89	98	65
" b	4.5	90	nd	55
human MBL a	5.6	88	59	23
" b	3.6	90	nd	34
rat RS	34	89	72	27
average values for entire table =		89	64	46

* Recovery of chromosomes calculated with respect to the number of mitotic cells collected at shake-off times the number of chromosomes in the mitotic cells of each culture.
** Cells swollen on ice.
***For clarification of abbreviated names of cultures, see Materials and Methods.

Chromosome recovery

Table 11.1 presents a summary of the recovery of mitotic cells and chromosomes observed during the isolation of chromosomes using the Hepes/$MgSO_4$ method. Chromosomes were isolated from a variety of in vitro cell lines, representing several mammalian species. Centrifugation of the cells harvested from the cultures after colchicine treatment results in a significant loss of mitotic cells. Approximately 36% of the mitotic cells are lost at this step in the procedure. After chromosome isolation, the average recovery of Chinese hamster chromosomes was 49% (range of five experiments, 25-78%). This recovery was calculated with respect to the number of mitotic cells in the original suspension and the number of chromosomes in the mitotic cells of this cell line. The chromosome recovery was approximately 28% for human fibroblast cells, 27% for rat rhabdomyosarcoma cells, 53% for the transformed human fibroblast line, and 60% for the transformed mouse fibroblast line.

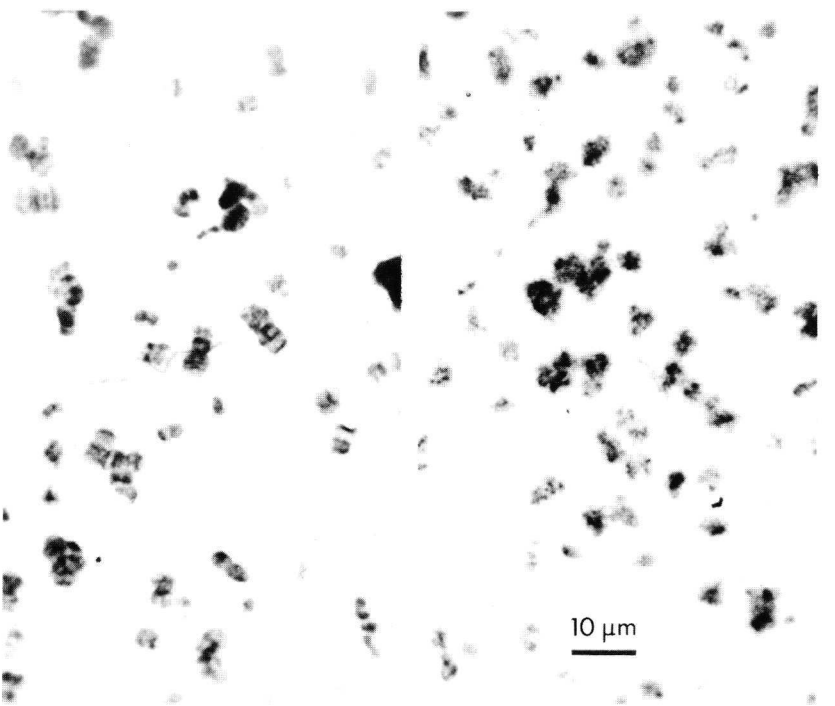


Figure 11.2. Photograph of representative slide showing Chinese hamster chromosomes attached to microscope slides according to the procedure in table 11.2A and stained according to the procedure in table 11.3A. The chromosomes in (A) were treated in suspension with dimethylsuberimidate (DMS). Those in (B) were not treated with DMS.

Banding

In order to obtain a banding pattern in isolated chromosomes, they must be attached first to slides with a preservation of morphology. They then must be subjected to staining conditions that produce bands.

Attempts to fix chromosomes isolated using the Hepes/$MgSO_4$ to slides using standard Carnoy's fixative (methanol:glacial acetic acid, 3:1 (v/v)) resulted in compact chromosomes that could not be banded. Centrifuging chromosomes directly onto slides in a cytocentrifuge also produced unsatisfactory results. The chromosomes became swollen and fell apart as the isolation buffer evaporated.

Chromosomes stabilized with the protein cross-linker dimethylsuberimidate (DMS) could be centrifuged on slides with retention of morphology. Figure 11.2 illustrates the effect of DMS on chromosome morphology. Without DMS treatment, the chromosomes decondense and are visible only as amorphous masses of chromatin. In contrast, the general morphology of chromosomes treated with DMS is preserved. Other fixatives, such as formaldehyde or glutaraldehyde, produced clumping and loss of morphology.

Table 11.2A

PROTOCOL FOR FIXING ISOLATED CHROMOSOMES TO SLIDES WITH PRESERVATION OF MORPHOLOGY

1. Spin 50 µl fetal calf serum onto slides in a cytocentrifuge at 250g for 30 s.
2. Spin a suspension of chromosomes treated with 3 mM DMS onto slides at 450g for 5 min.
3. Immediately thereafter, spin 100 µl of 96% ethanol onto slides at 450g for 1 min.

Table 11.2B

STAINING PROCEDURE FOR THE PRODUCTION OF BANDS IN ISOLATED CHROMOSOMES FIXED TO SLIDES

1. Rinse slides in Carnoy's fixative.
2. Incubate slides in 2xSSC (2xSSC = 0.3 M NaCl + 0.03 M sodium citrate) for 5 min at 60°C.
3. Rinse slides quickly in tap water.
4. Incubate slides in a 0.1% trypsin solution in phosphate buffer at pH 6.8 for 10 s at room temperature.
5. Rinse slides quickly in tap water.
6. Stain preparations in 3% Giemsa stain solution in phosphate buffer, pH 6.8, for 10 min.
7. Dry preparations and observe under light microscope.

To increase the number of chromosomes that could be attached to slides and to further improve their morphology, several steps were added to the procedure. Table 11.2A outlines the protocol that resulted in optimal chromosome attachment. More chromosomes could be attached to slides precoated with fetal calf serum than to clean slides. With clean slides, the chromosomes moved during centrifugation and were absorbed in the absorbent filter paper. The optimal amount of fetal calf serum was determined to be 50 µl. The speed used to centrifuge the chromosomes onto slides is also important for attachment. At slow rates (less than 130g), chromosomes apparently moved together and formed clumps during the drying procedure. At higher speeds (greater than 300g), a high frequency of single chromosomes was found on the slides. Additional fixation of

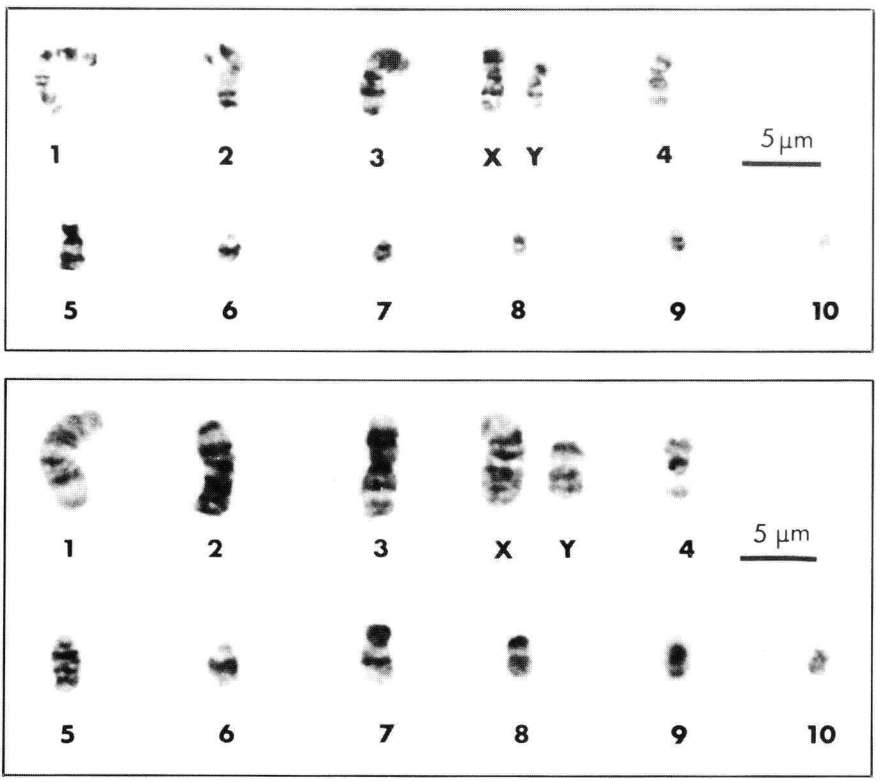


Figure 11.3. Chinese hamster chromosomes isolated using the Hepes/$MgSO_4$ procedure, fixed with DMS, and attached and stained on slides as described in table 11.2. These karyograms were compiled from photographs taken of two separate slides.

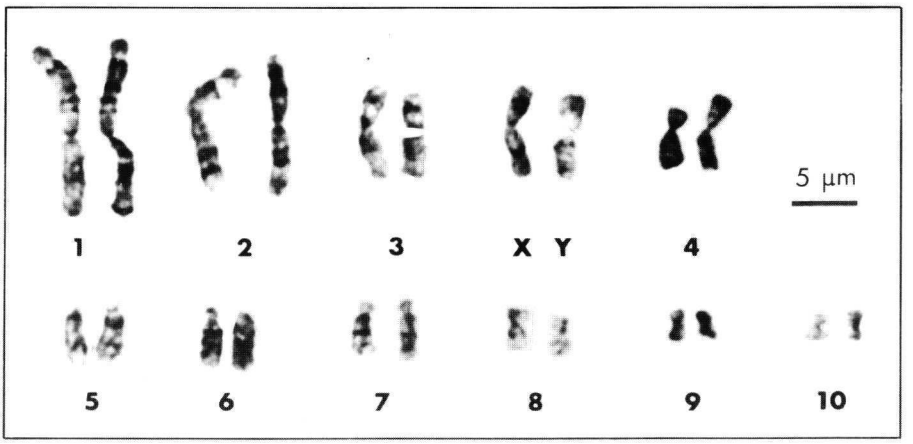


Figure 11.4. Karyogram of chromosomes cut from a photograph of one mitotic cell in a conventional metaphase cell preparation. The chromosomes were banded according to the procedure in table 11.2B.

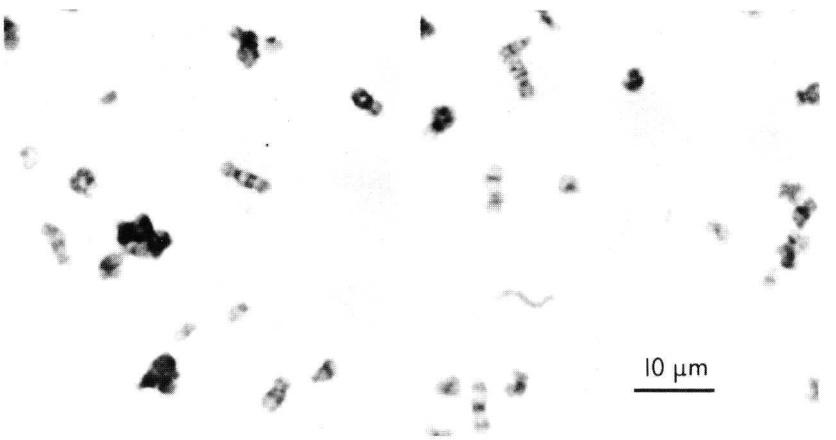


Figure 11.5. Photograph of two representative slides showing the banding pattern in human chromosomes, which have been isolated in suspension. The chromosomes were cross-linked with DMS, fixed to slides as described in table 11.2A and stained according to the procedure in table 11.2B.

chromosomes with 96% ethanol, immediately after they were spun onto the slides, also resulted in improved chromosome morphology. This effect was observed also with chromosomes not treated with DMS.

The staining procedure that gave optimal results in producing a banding pattern in the isolated chromosomes is outlined in table 11.2B. This is a modification of the method described by Gallimore and Richardson (1973). The chromosomes on the slides are incubated at 60°C in high salt (2xSSC=0.3 M NaCl, 0.03 M sodium citrate), digested with trypsin, and subsequently stained with Giemsa. Rinsing the slides with Carnoy's fixative was found to be essential for the production of bands.

This staining procedure can be used to band chromosomes after their isolation into suspension, as well as chromosomes in conventional metaphase spreads. Figure 11.2A shows a representative photograph of isolated Chinese hamster chromosomes, stained on slides after DMS treatment and attachment. The banding pattern is sufficient for the identification of the chromosomes. Figure 11.3 shows two karyograms compiled from photographs like the one in figure 11.2A. Some variation in the degree of chromosome swelling is evident from these two karyograms. Despite this variation, the majority of chromosomes on the slides can be identified by their banding pattern.

The staining procedure can also be used to band the chromosomes in conventional metaphase cell preparations. Figure 11.4 shows a typical karyogram from a Chinese hamster metaphase cell.

Human chromosomes isolated into suspension using the Hepes/$MgSO_4$ procedure can also be banded according to the protocols in tables 11.1A and B. Figure 11.5 shows a representative photograph of banded human chromosomes. Approximately 50% of the human chromosomes can be identified in photographs such as this (P.L. Pearson, personal communication).

Isolated chromosomes stained in suspension with Hoechst 33258 and chromomycin A3 and treated with sodium citrate can also be banded satisfactorily using this procedure (not shown).

DISCUSSION

The results in this chapter show that DNA fragments extracted from chromosomes prepared with the Hepes/$MgSO_4$ method are larger than 50 kbase pairs (greater than 32 MDalton) and are of sufficient size for most recombinant DNA techniques (Maniatis et al., 1982). These DNA fragments are similar in size to the fragments isolated from the chromosomes prepared using several other methods. These observations confirm previously published results (Hepes/$MgSO_4$: 20 to greater than 50 kb, chapter 7; 20 to greater than 50 kb after high speed sorting, Peters et al., 1985)

(hexylene glycol procedure: 30 MDalton, Yu et al., 1981; and 10-100 MDalton after high speed sorting, Gray et al., 1981). Using alkaline sucrose gradients, Blumenthal et al. (1979) have reported molecular weights of 200 MDalton and 2 MDalton for DNA isolated from the chromosomes prepared using the polyamine buffer and those prepared in hexylene glycol, respectively. In the experiments reported here, the molecular weight determined in agarose gels of the DNA from the polyamine procedure is similar to that of the other methods.

Chromosomes isolated using the Hepes/$MgSO_4$ procedure, stained with fluorochromes and treated with sodium citrate, can be banded. The banding patterns produced are sufficient for the identification of the majority of human and Chinese hamster chromosomes. This indicates that internal structures in the chromosomes, required for the production of bands, are preserved during the isolation procedure. Experiments are in progress to determine whether chromosomes sorted using a flow cytometer can also be banded in this way.

An important part of the procedure for fixing and staining chromosomes on slides to produce bands is the prevention of chromosome disintegration during the drying procedure. Dimethylsuberimidate treatment of chromosomes stabilizes the chromatin structure and allows retention of normal morphology during the banding procedure. This substance can also be used to stabilize nuclei and chromosomes for experiments in which the DNA must be denatured (chapters 13,14).

Other banding protocols were tested and produced no, or only vague bands. Those tested include a trypsin/Giemsa procedure (Pearson and van Egmond-Cowan, 1976), an acetic/saline/Giemsa (ASG) technique (Sumner et al., 1971), an acridine orange technique (Dutrillaux, 1973), a quinacrine technique (Yu et al., 1981), and a basic fuchsin technique (Scheres and Merckx, 1976; van Prooijen-Knegt, 1983).

Recently developed techniques may make it possible to circumvent banding analysis for the identification of chromosomes. Individual chromosome types, discriminated in a flow cytometer, can be sorted onto nitrocellulose filters. The hybridization of chromosome-specific DNA sequence probes to these filters can be used to identify the chromosomes responsible for peaks in the fluorescence distribution (Bernheim et al., 1983; Collard et al., 1985). If contaminating chromosomes are of one type, they can be detected in this way at a frequency greater than 10% (Bernheim et al., 1983). Preliminary results indicate that this technique can also be applied to chromosomes isolated using the Hepes/$MgSO_4$ procedure (chapter 15).

The results shown here indicate that the Hepes/$MgSO_4$ procedure meets many of the criteria for an ideal isolation protocol for studies in

which chromosomes are sorted for subsequent analysis. Human chromosomes can be well resolved in a flow karyotype (chapter 7) and low staining variability is observed (chapter 8). The molecular weight of DNA isolated from the chromosomes is high, the chromosome recovery from original cells is high, and the chromosomes can be banded. This procedure is currently being used in the joint Los Alamos/Livermore project for the construction of chromosome-specific genomic libraries from sorted chromosomes.

Chapter 12

IMMUNOFLUORESCENT DETECTION OF HISTONE 2B ON METAPHASE CHROMOSOMES USING FLOW CYTOMETRY*

SUMMARY

A monoclonal antibody against histone 2B was used as a reagent to stain isolated chromosomes for analysis using flow cytometry. Chromosome suspensions were treated with a mouse monoclonal antibody specific for the histone 2B (clone HBC-7) and then with a fluorescein labeled goat anti-mouse-IgM antibody. The chromosomes were also stained for DNA content with either Hoechst 33258 or propidium iodide. The amount of antibody and the amount of DNA-specific stain bound to each chromosome were measured simultaneously using flow cytometry. The order of the steps in the staining protocol is important. Propidium iodide prevents this anti-histone 2B antibody from binding to chromosomes, and therefore must be added only after antibody labeling is completed. The addition of Hoechst 33258 before antibody labeling reduces antibody binding by only 20-30%. Binding of the anti-histone-2B antibody was proportional to the DNA content of both human and Chinese hamster chromosomes. Human chromosomes bind an average of three to four times as much of this anti-histone-2B antibody as do Chinese hamster or mouse chromosomes of the same DNA content. This was determined by analyzing mixtures of human and Chinese hamster chromosomes and human and mouse chromosomes. The results demonstrate that it is possible to label the proteins of chromosomes in suspension with fluorescent antibodies and to use these reagents for the analysis of chromosome structure by flow cytometry.

INTRODUCTION

Flow cytometry provides a means for rapid quantification of the fluorescence of individual mammalian chromosomes (Gray et al., 1975b,

*This chapter has been adapted from a paper published by Barb Trask, Ger van den Engh, Joe Gray, Marty Vanderlaan, and Bryan Turner, in Chromosoma 90:295-302 (1984).

1979b). Emphasis in the past has been on using DNA-specific fluorescent stains as cytochemical probes. For this chapter, a monoclonal antibody was used to fluorescently label a structural protein in isolated chromosomes for subsequent flow cytometric analysis.

For flow cytometry, suspensions of chromosomes are prepared by swelling mitotic cells in a hypotonic solution and mechanically rupturing the cells in the presence of a detergent. The chromosomes are usually stained with at least one DNA-specific fluorescent dye. Two widely used stains are Hoechst 33258 (HO), a non-intercalating DNA stain that binds principally to adenine-thymidine (A-T) base pairs, and propidium iodide (PI), an intercalating DNA stain with little or no base pair specificity. The fluorescent signals from individual chromosomes are measured as the chromosomes pass through the laser beam of a flow cytometer. Hundreds of chromosomes can be analyzed each second, so that a frequency distribution of the fluorescence intensity of a chromosome population can be measured rapidly. Different chromosomes can be distinguished on the basis of total DNA content or of base pair ratios. When a single DNA stain such as HO or PI is used, approximately 15 groups of human chromosomes can be resolved in a univariate chromosome distribution (flow karyotype). This technique can be used for quantitative flow karyotyping (Gray et al., 1975b, 1979b; Young et al., 1981) and for purification of individual chromosome types for identification or biochemical analysis (Carrano et al., 1979b; Davies et al., 1981).

A procedure to obtain metaphase chromosomes free in suspension for flow cytometric analysis is described in chapter 6. This procedure routinely yields chromosome suspensions with low levels of small particle debris and few chromosome clumps. High resolution flow karyotypes have been obtained from Chinese hamster cell lines and from several different human cell types (chapter 7). The chromosomes that are prepared using this isolation procedure are stable in suspension. Unstained chromosomes can be stored for several weeks. Furthermore, the chromosomes tolerate a moderate amount of gentle centrifugation. The stability of the chromosomes and their low tendency to clump following centrifugation make it possible to subject chromosomes to procedures that require several steps separated by washing and centrifugation such as those required for labeling with fluorescently tagged monoclonal antibodies.

The monoclonal antibody used in this study is specific for the chromosome structural protein, histone 2B, and has been described by Turner (1981, 1982). An octameric complex of histone 2B and the other core histones (2A, 3 and 4) serves as a core around which the DNA is wound, forming the basic unit of chromatin structure, the nucleosome (McGhee and Felsenfeld, 1980). Polyclonal antibodies to the histones have been used to

probe nucleosomal structure (Bustin et al., 1978; Silver et al., 1978). The monoclonal anti-histone 2B antibody used in this study is an IgM and was produced by fusion of spleen cells from a mouse immunized with human lymphoblast chromatin with mouse myeloma cells. This monoclonal antibody has been shown to bind to metaphase chromosomes and interphase nuclei of fixed cells on slides. The specificity of this antibody to histone 2B has been established by demonstrating its binding to the histone 2B band (or bands) on polyacrylamide gels and to pure histone 2B by radioimmunoassay (Turner, 1982; Whitfield et al., submitted). Histones of the nucleosome have been highly conserved throughout evolution and immunological reagents that label histones can be expected to have considerable cross-reactivity among different species. Indeed, fluorescence microscopy has shown that the monoclonal antibody binds to fixed chromosomes from a variety of human and rodent cells (Turner, 1982).

This paper describes the results of experiments designed to determine whether the binding of anti-H2B to chromosomes could be measured using flow cytometry. Since histone 2B is an integral part of each chromosome, an antibody directed against it was of interest primarily to test the possibility of staining chromosomes in suspension with immunological reagents and to ascertain the best staining protocol. As an adjunct to this, the effect of propidium iodide and Hoechst 33258 on anti-H2B binding was investigated.

MATERIALS AND METHODS

Cell cultures

Chromosomes were prepared from a culture derived from the M3-1 Chinese hamster cell line, from a human diploid fibroblast culture, LLL (Lawrence Livermore Laboratory) strain 811, from foreskin, and from a suspension culture derived from a mouse myeloma line (a gift from Dr. L.A. Herzenberg, Stanford, CA). All three cell lines were maintained in culture as described in chapter 7. The Chinese hamster and human cells were blocked in mitosis at a cell density of approximately 75% confluency. Growth medium was removed and replaced with fresh growth medium containing fetal calf serum (FCS) and 0.1 μg/ml colcemid (Sigma, St.Louis). The mouse myeloma cells were blocked by addition of 0.1 μg/ml colcemid directly to the flask containing dividing cells. Chinese hamster cells were blocked for 4 h. Mouse and human cells were blocked for 10-12 h. Mitotic cells were shaken from Chinese hamster and human cultures. This enrichment of mitotic cells was not possible with mouse suspension cultures.

Preparation of chromosome suspensions

Chromosomes were isolated from mitotic cell suspensions for flow cytometry as described in chapter 6. Aliquots containing approximately 10^5 mitotic cells were centrifuged and resuspended in 1 ml hypotonic swelling buffer. Dithiothreitol, a usual constituent of the swelling buffer, was eliminated as it was expected to reduce antibody stability. For some experiments, the DNA stain, either propidium iodide (PI) or Hoechst 33258 (HO), was added after syringing and before incubation at 37°C for RNA digestion.

Antibody labeling of chromosomes

The isolation of the monoclonal antibody to histone 2B (anti-H2B) was reported by Turner (1981, clone HBC-7). The antibody was produced by fusion of spleen cells from a mouse immunized with human lymphoblast chromatin with the mouse myeloma cell line (Ag8.653). Ascites fluid containing this antibody was centrifuged in an Eppendorf centrifuge (model 5414) before use to remove aggregates. Aliquots of 50 µl of a 1:30 anti-H2B dilution were added to 500 µl chromosome suspension (final anti-H2B dilution, 1:300) to which FCS had been added to minimize nonspecific antibody binding (final FCS concentration= 2%). Dilutions of antibody were made in phosphate buffered saline (PBS). PBS was added to control samples that received no anti-H2B. After 15 min at room temperature, the suspension was spun at 300-400g for 10 min. The supernatant fluid was removed, and the pellet was loosened. The chromosomes were resuspended in 500 µl chromosome isolation buffer containing 0.25% Triton X-100 and 2% FCS. A 1:30 dilution of the fluorescein-conjugated IgG fraction of goat anti-mouse IgM (mu chain specific) (Cappell Labs, Cochranville, PA) was spun in an Eppendorf centrifuge before use. 50 µl of the 1:30 anti-IgM-FITC was added to each 500 µl chromosome sample (1:300 final dilution). After 15 min at room temperature, the chromosomes were gently added to tubes on top of a 1 ml layer of chromosome isolation buffer containing 10% FCS. These tubes were centrifuged for 10 min at 300-400g. The supernatant fluid was removed and 500 µl chromosome isolation buffer was added. In most experiments, the chromosomes were stained at this point with either 29 µM propidium iodide or 2.7 µM Hoechst 33258 (both from Sigma). The chromosomes were gently syringed three times through a 22 1/2-gauge needle, and kept on ice until they were analyzed using flow cytometry.

Flow cytometric analysis

Chromosome suspensions were analyzed using the LLL dual beam cytometer (Dean and Pinkel, 1978) equipped with two Spectra-Physics (Mountain View,

CA) lasers (model 171 for excitation in the ultraviolet and model 164 in the visible wavelengths). In this system, chromosomes were forced to flow one-by-one in a sheath of distilled water through one or two laser beams. Both lasers were utilized when HO was used as the DNA stain. Only one laser was needed when PI was used. To measure Hoechst 33258 fluorescence, chromosomes were illuminated with a laser emitting 1 W power of multiline UV light (351 and 364 nm). The fluorescence passed through a Ditric Optics (Hudson, MA) 425 nm high pass filter to block UV light and onto a photomultiplier that produced an electrical signal proportional to the fluorescence intensity. Alternatively, a laser tuned to 488 nm at 800 mW was used to excite propidium iodide. Its fluorescence was measured through a Corning 2-63 long pass filter (passing wavelengths $\geq$590 nm). Fluorescein fluorescence was measured by exciting with a laser tuned to 488 nm at 800 mW power. Fluorescein fluorescence passed through a 514.5 nm band pass filter (03-FIL-021) and onto a second photomultiplier. The signals for both photomultipliers were integrated and amplified. For univariate distributions, the fluoresence intensity of the DNA stain on each chromosome was stored in the 256 channel memory of a pulse height analyzer (ND 620, Nuclear Data, Schaumberg, IL). For bivariate plots, the intensity of DNA-specific dye fluorescence (PI or HO) and the intensity of FITC fluorescence of each chromosome were stored in the 64 by 64 matrix of the pulse height analyzer. Chromosomes were analyzed at a rate of 200 chromosomes/s. The fluorescent intensities of a large number of chromosomes (usually 10^5) were accumulated to form a bivariate distribution with fluorescence due to the DNA stain on the abscissa and fluorescence due to FITC on the ordinate.

HPLC analysis of histones

The histone content of chromosomes was assayed using a high performance liquid chromatography procedure developed by Mazrimas and Balhorn (1983). Histone analyses were made of isolated chromosomes before and after propidium iodide staining as well as of stained chromosomes that had been purified by sorting to eliminate cytoplasmic contaminants. Briefly, isolated chromosomes were concentrated by centrifugation and dissolved in 3 M guanidine hydrochloride. The proteins were separated from the DNA on a gel permeation column (3TSK-3000 SW columns in tandem) and lyophilized. The protein residue was then dissolved in 0.1% trifluoracetic acid and analyzed on a 10 um PRP-1 column. All histones and the high mobility group proteins were resolved by this technique.

RESULTS

When suspensions of Chinese hamster or human chromosomes are stained with DNA-specific fluorescent dyes and are analyzed by flow cytometry, unique fluorescence distributions are observed for the two species. This is illustrated in figure 12.1. The fluorescence intensity distributions of chromosomes isolated from the human fibroblast strain 811 and stained with either propidium iodide (PI) or Hoechst 33258 (HO) are shown in figures 12.1a and c. Figure 12.1b and d show HO and PI distributions for chromosomes from the Chinese hamster M3-1 cell line used in this study. Different fluorescence distribution profiles are obtained with HO and PI due to the fact that PI binds to all DNA base pairs, and HO binds

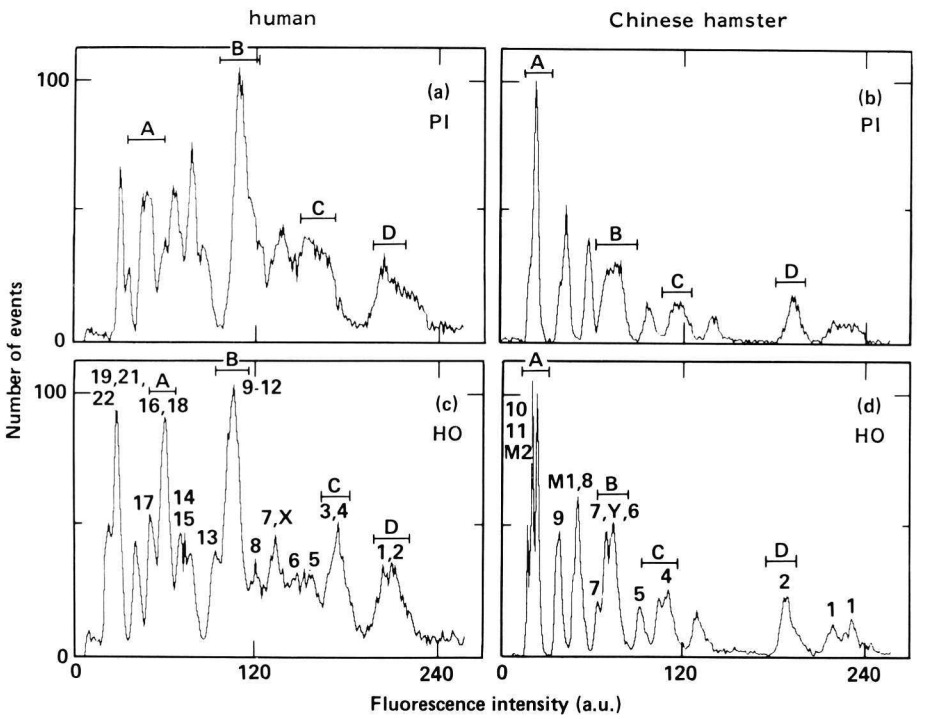


Figure 12.1. Histograms of Hoechst 33258 (HO)-labeled chromosomes from human cells (LLL 811) and the Chinese hamster cells used in this study and propidium iodide (PI)-labeled chromosomes from the same cell lines. The numbers above the peaks indicate the chromosomes that are responsible for the peaks. The letters identify major peaks to aid in their identification in subsequent figures. A) Human chromosomes stained with PI; B) Chinese hamster chromosomes stained with PI; C) Human chromosomes stained with HO; D) Chinese hamster chromosomes stained with HO.

principally to adenine-thymidine base pairs. The fluorescence distribution with a given dye is highly reproducible and is characteristic for each cell line. In each of the histograms shown, the amplifier gain was adjusted to spread the fluorescence intensity signals over the full range of a 256 channel distribution. The gain was approximately two times higher for human chromosomes than for Chinese hamster chromosomes to compensate for the fact that human chromosomes are on average about half as large, and therefore half as fluorescent, as Chinese hamster chromosomes. Some of the major peaks in the histograms in figure 12.1 have been labeled with letters to aid in the interpretation of subsequent figures. The numbers above the peaks indicate the chromosomes expected to be responsible for each peak. These chromosome assignments are based on previous studies in which the chromosomes in each peak were sorted and Q-banded for identification (Gray et al., 1975b; Yu et al., 1981).

The binding of the monoclonal antibody specific for histone 2B (anti-H2B) to Chinese hamster and human chromosomes is shown in figure 12.2. Chromosomes of both species were treated with either anti-H2B (top panels) or with PBS (bottom panels). They were subsequently incubated with anti-IgM-FITC and then stained with HO. In the bivariate distributions shown in figure 12.2, the fluorescence of the HO bound to each chromosome is plotted along the X-axis. The Y-axis gives the intensity of the FITC fluorescence, which is an indirect measure of the amount of bound anti-H2B. For each species, both control and anti-H2B-treated chromosomes were analyzed at the same photomultiplier and amplifier settings. The relative fluorescence intensities of the two species are not directly comparable in this figure, as the signal amplification was adjusted to expand the distributions to full scale in both the X and Y directions for each species.

The data in the bivariate plots in figure 12.2a and d have been collapsed onto the abscissa to yield the univariate distributions of HO fluorescence intensity in panels c and f. The resolution of these distributions is lower than that of the distributions in figure 12.1. Part of this difference can be ascribed to the fact that ther are only 64 channels along each axis in the bivariate plot, compared to 256 channels in figure 12.1. It is also due to some deterioration in the chromosome suspension quality after antibody labeling and centrifugation. Despite the loss of resolution, the major chromosome peaks can still be identified and are labeled as in figure 12.1.

For both human and Chinese hamster chromosomes, the fluorescence intensity of chromosomes treated with anti-H2B is well above background fluorescence levels (the fluorescence of chromosomes treated with only

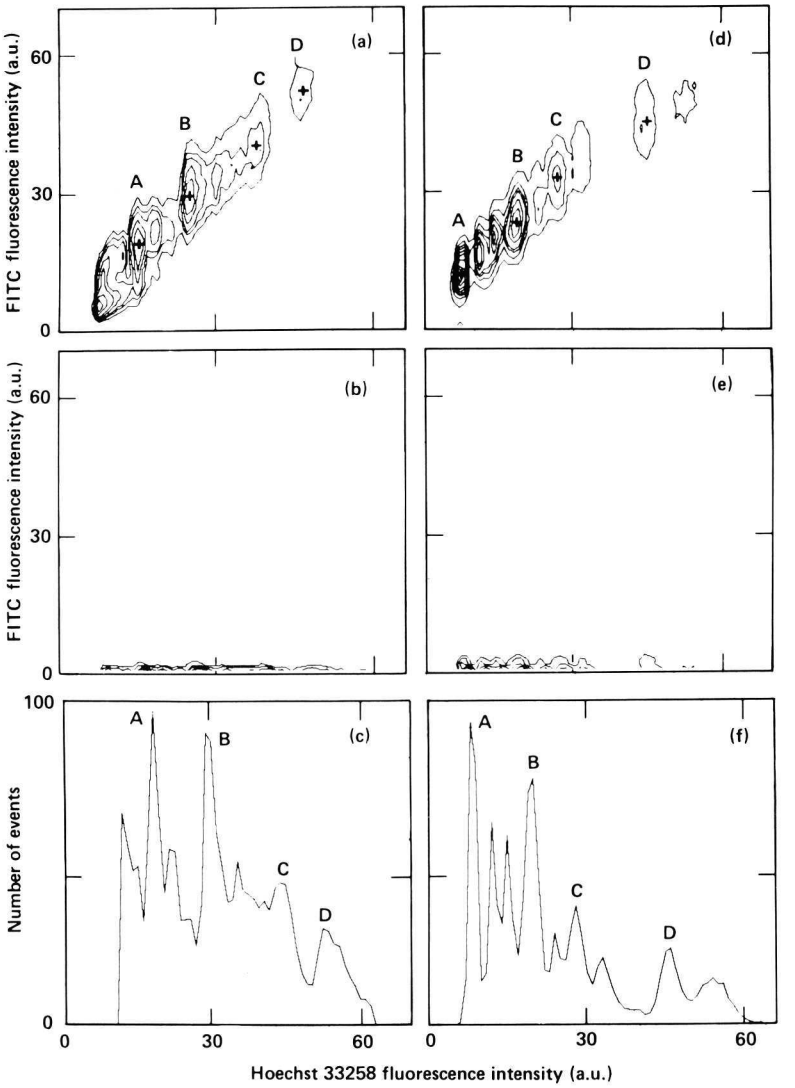


Figure 12.2. Effect of labeling Chinese hamster and human chromosomes with anti-H2B. Chromosomes shown in the top panels (a, d) have been treated with anti-H2B, followed by treatment with anti-IgM-FITC. Chromosomes shown in the middle panels (b, e) have been stained with anti-IgM-FITC only. In the bottom panels (c, f), the data from the top panels have been collapsed onto the abscissa. The major chromosome peaks are labeled to correspond with those in figure 12.1. The panels in the left column (a-c) show measurements on human chromosomes at the same photomultiplier and amplifier settings. The panels in the right column (d-f) show measurements on Chinese hamster chromosomes. These chromosomes were measured at different photomultiplier and amplifier settings than the human chromosomes.

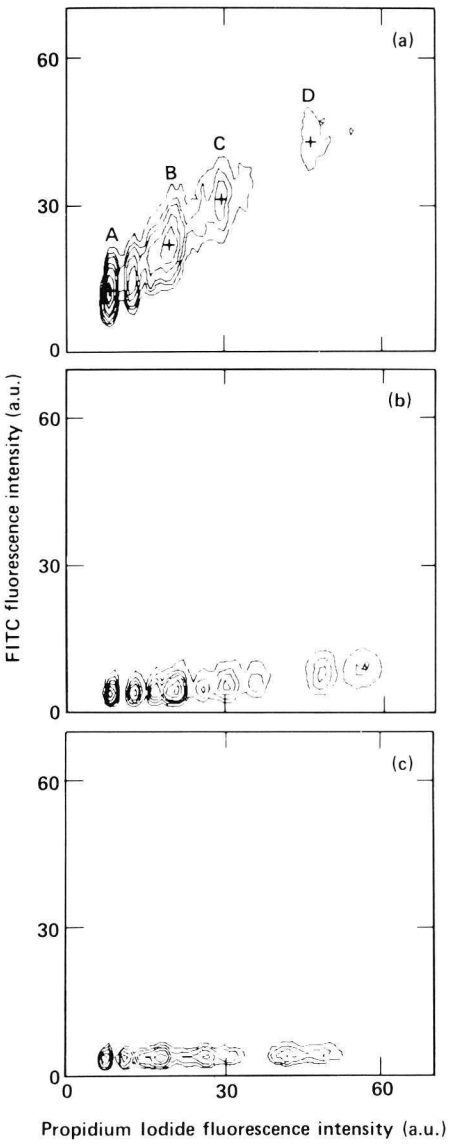


Figure 12.3. Effect of propidium iodide (PI) on anti-H2B binding to Chinese hamster chromosomes. a) PI staining after antibody labeling; chromosomes labeled with both anti-H2B and anti-IgM-FITC. b) PI staining after chromosome isolation, but before antibody labeling; chromosomes labeled with both anti-H2B and anti-IgM-FITC. c) chromosomes labeled with anti-IgM-FITC only; PI staining after antibody labeling. All three panels were measured at the same photomultiplier and amplifier settings. Labels on the major peaks correspond with figure 12.1

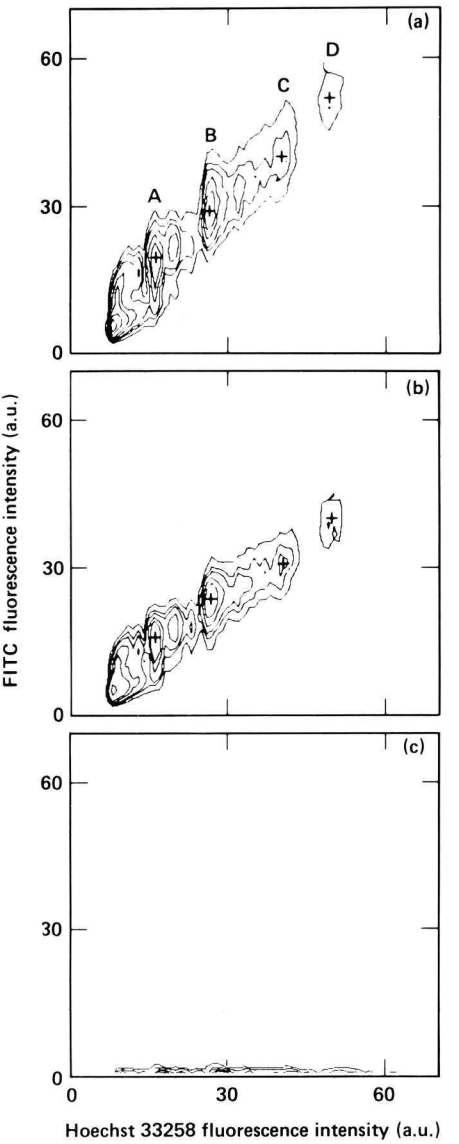


Figure 12.4. Effect of Hoechst 33258 (HO) on anti-H2B binding to human chromosomes. a) HO staining after antibody labeling; chromosomes labeled with both anti-H2B and anti-IgM-FITC. b) HO staining after chromosome isolation, but before antibody labeling; chromosomes labeled with both anti-H2B and anti-IgM-FITC. c) chromosomes labeled with anti-IgM-FITC only; HO staining after antibody labeling. All three panels were measured at the same photomultiplier and amplifier settings. The major peaks are labeled to correspond with figure 12.1.

anti-IgM-FITC). Anti-H2B binding to the chromosomes of either species is proportional to the amount of HO bound to the chromosomes.

Similar results were obtained when PI was used to stain the chromosomes for total DNA content. The timing of PI addition, however, greatly influences antibody binding to chromosomes. This is shown in figure 12.3 for Chinese hamster chromosomes. All three plots were made at the same photomultiplier and amplifier settings. If chromosomes are stained with PI before they are exposed to anti-H2B, little specific antibody binding can be demonstrated. This is also the case for human chromosomes. High performance liquid chromatographic analysis of the proteins extracted from unstained and PI-stained chromosomes showed that the PI staining did not remove the histones.

Staining of the chromosomes with HO before antibody labeling results in only a slight reduction in anti-H2B binding. This is shown in figure 12.4 for human chromosomes. The three plots were made at the same photomultiplier and amplifier settings. Antibody binding to chromosomes prestained with HO is 20-30% less than antibody binding when HO is added after the antibody labeling procedure. This is the case for both human and Chinese hamster chromosomes.

During these experiments, it was noticed that the fluorescence signals due to bound antibody were more intense for human chromosomes than for Chinese hamster chromosomes. This was investigated using a mixture of human and Chinese hamster chromosomes. The mixture was stained with anti-H2B, followed by incubation in anti-IgM-FITC. A mixture of human and Chinese hamster chromosomes treated with only anti-IgM-FITC served to determine background binding. The chromosome mixtures were stained with HO after antibody labeling was completed. Figure 12.5 shows the HO fluorescence vs. FITC fluorescence distributions for these mixtures. HO staining of the mixture allows identification of the lines of clusters as either human or Chinese hamster chromosomes. The line of chromosome clusters with high FITC fluorescence are recognizable as human by their distribution pattern along the HO axis. The clusters of chromosomes with low FITC fluorescence are the Chinese hamster chromosomes in the mixture. This was repeated over a period of time on five different human/Chinese hamster mixtures. Human chromosomes bind about three times (range: 2.5-5.5 times) more anti-H2B than Chinese hamster chromosomes of the same HO or PI fluorescence.

A difference in the relative amount of antibody binding was also observed between human and mouse chromosomes. A mixture of human and mouse chromosomes was incubated with either anti-H2B or with PBS, followed by treatment with anti-IgM-FITC. As above, the chromosomes were stained with HO after the centrifugation step to remove free anti-IgM-FITC.

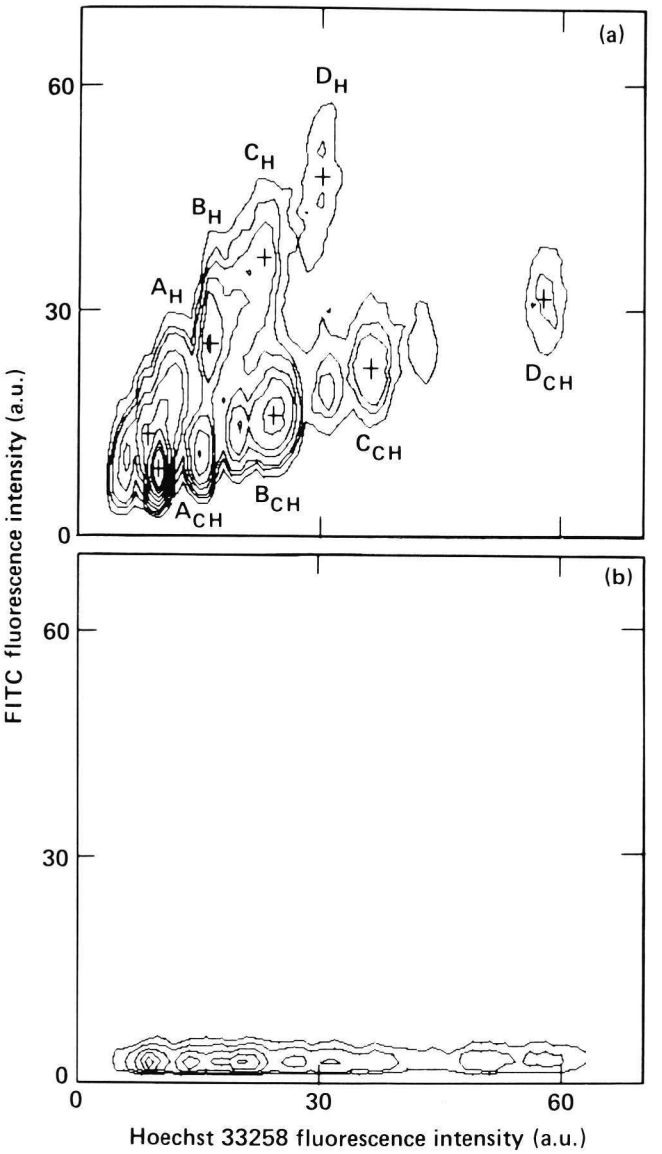


Figure 12.5. Mixture of Chinese hamster and human chromosomes labeled with anti-H2B and Hoechst 33258. a) Mixture labeled with anti-H2B and anti-IgM-FITC. b) Mixture labeled with anti-IgM-FITC only. Both panels were measured at the same photomultiplier and amplifier settings. A(H)-D(H) denote major human chromosome peaks; A(CH)-D(CH) identify major Chinese hamster peaks corresponding to the labeling in figure 12.1.

The results of the flow cytometric analysis of these mixtures are shown in figure 12.6. Again, the clusters of chromosomes along the diagonal are recognizable as human chromosomes by their HO distribution pattern. Human chromosomes have a FITC fluorescence intensity more than 4 times as high as mouse chromosomes with the same HO fluorescence. This indicates that more than 4 times as much anti-H2B binds to human chromosomes than binds to mouse chromosomes of comparable size.

DISCUSSION

Chromosomes in suspension can be labeled with antibodies directed against structural proteins. The amount of antibody bound to chromosomes of different DNA content can be quantified rapidly in a flow cytometer. The procedures described in this paper open the possibility that the protein component of individual chromosomes can be studied with a wide range of fluorescently labeled probes. In these experiments, an antibody against histone 2B was used (anti-H2B). The anti-H2B molecules bound to chromosomes were made visible with an anti-IgM conjugated to FITC. The DNA of the chromosomes was stained with a suitable fluorescent DNA-specific dye. Significant binding of anti-H2B was obtained for human, mouse and Chinese hamster chromosomes.

Although the antibody labeling procedure causes some deterioration in the resolution of the DNA distributions, most chromosome groups can still be resolved. Human and Chinese hamster chromosomes can be distinguished from one another.

Within a species, anti-H2B does not distinguish between different chromosomes. For each species, the amount of antibody binding is roughly proportional to the DNA content of each chromosome. This is consistent with what is known about the association of histones and the DNA double helix in eucaryotic chromosomes.

Observations on the interaction between propidium iodide (PI) and anti-H2B binding suggest that the superstructure of the chromosomes may determine the degree of antibody binding. Little specific antibody binding is found if the DNA is stained with PI before labeling with anti-H2B. If PI is to be used as the DNA stain, it must be added only after antibody labeling has been performed. Hoechst 33258 (HO) interferes to a lesser extent with antibody binding to chromosomes. HO can be added before or after antibody labeling has taken place.

Propidium iodide stains DNA by intercalating between the two DNA strands in the double helix. In so doing, it causes a significant conformational change in the DNA helix (Cantor and Schimmel, 1980). It has been determined that a DNA helix fully saturated with ethidium bromide (a

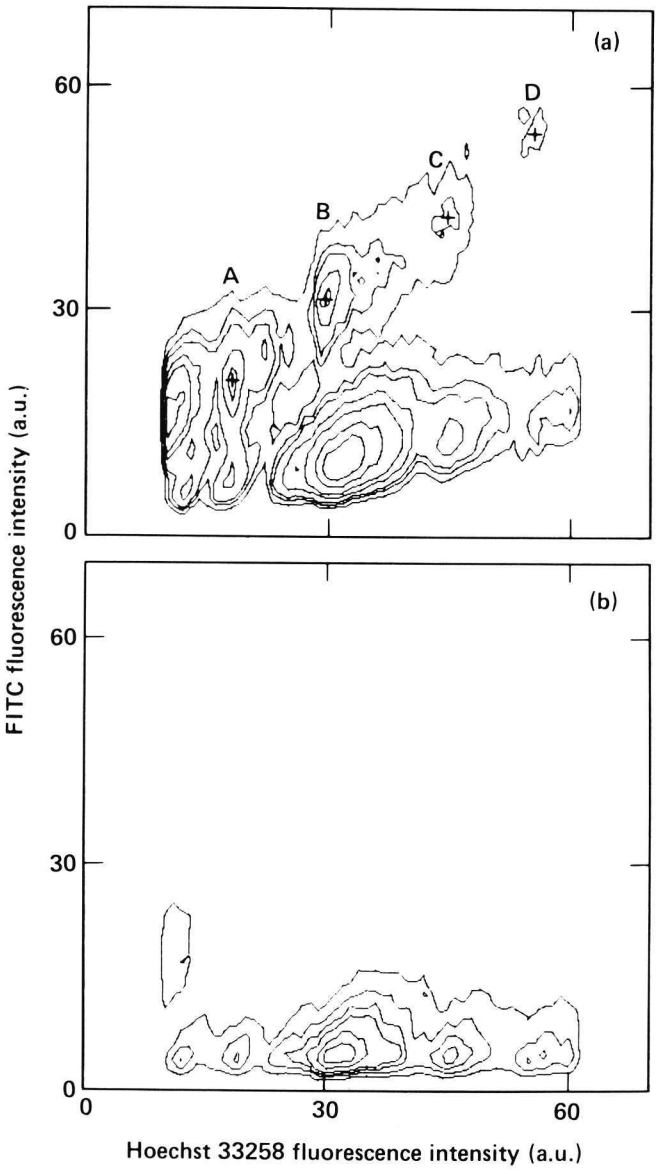


Figure 12.6. Mixture of mouse and human chromosomes labeled with anti-H2B and Hoechst 33258. a) Mixture labeled with anti-H2B and anti-IgM-FITC. b) Mixture labeled with anti-IgM-FITC only. Both panels measured at the same photomultiplier and amplifier settings. A-D identify the major human chromosome peaks to correspond with the labeling in figure 12.1.

dye similar to PI) increases 50% in length. It is probable that changes in the DNA/histone association and changes in the superhelical configuration in the chromosome occur when PI intercalates in the DNA double helix. The dimensions of the nucleosome and the antibody molecules are such that steric hindrance could account for the observed reduction in antibody binding. The nucleosome measures 10 nm in diameter with the histones located inside a a spiral of 2 nm wide DNA strands. The nucleosomes themselves are thought to be packed in fibers 20-30 nm in diameter (Worcel, 1978). In comparison, the antigen-combining site of an IgM antibody is a 4 nm diameter arm on a protein measuring 30 nm in diameter (Sell, 1980). It is conceivable that even slight conformational changes in the DNA-histone complex or in the packing of nucleosomes due to the incorporation of PI in the DNA strands could interfere with antibody binding. Analyses of the histone content of sorted chromosomes by high performance liquid chromatography excludes the possibility that PI treatment removes the histones from the chromosomes. Hoechst 33258 binds to the DNA strands without intercalating (Comings, 1975). It presumably has a different effect, if any, on DNA conformation and, for this reason, affects the accessibility or the affinity of the antibody to histone 2B to a lesser degree than PI.

Flow cytometric analysis of the fluorescence intensity of chromosomes labeled with the anti-histone 2B antibody reveals species differences in the amount of antibody bound per unit DNA. Approximately three times more anti-H2B binds to human chromosomes than binds to Chinese hamster or mouse chromosomes of the same DNA content.

It is possible that the differential binding is caused by differences among species in the composition of the histone 2B molecule. Histone 2B is known to vary slightly among species, primarily in the lysine-rich N-terminal region of the molecule that associates with DNA (Von Holt et al., 1979; Spiker and Isenberg, 1978; Elgin et al., 1979). The monoclonal antibody used in this study was produced by immunization with human chromatin and is directed against a site within 20 amino acid molecules of the N-terminus (Turner, 1982; Whitfield et al., submitted). However, available sequence data provide no evidence for differences in the N-terminal regions of human and rodent histone 2B (Ohe et al., 1979; Martinage et al., 1979). Although slight differences in the number of base pairs in the spacer unit of rodents and human chromosomes do exist (Compton et al., 1976), differences in the absolute number of histone 2B molecules per chromosome appear too small to explain the large difference in antibody binding between species.

If the antibody is sensitive to differences in the degree of phosphorylation, acetylation or other post-translational modification of

the histones, and these differences exist between human and rodent chromosomes, this could be the basis of the species difference in the binding of anti-H2B.

Another explanation for the species difference in antibody binding may be that there exist conformational differences in the supercoiling and packing of nucleosomes in the mitotic chromosomes of different species. A larger number of histone 2B molecules may be accessible for the IgM antibody in human chromosomes than in Chinese hamster or mouse chromosomes.

If steric hindrance is the cause of the effect of PI on antibody binding and/or of the observed species differences in binding, antibodies to chromosome components may be useful in studying the superstructure of chromosomes. It is of interest to determine whether the same effects are observed with antibodies against other histones, other antigenic sites on histone 2B or with antibody molecules such as IgG, which are smaller than IgM molecules.

The binding of anti-H2B to chromosomes of a given species is proportional to DNA content. It does not bind preferentially to certain chromosomes. This fact limits its usefulness as an additional tool in distinguishing the many human chromosomes from each other using flow cytometry. However, the ability of the monoclonal antibody to differentiate between human and Chinese hamster chromosomes and between mouse and human chromosomes could prove useful. Hybrids of human and Chinese hamster cells are being produced to study which human chromosomes are necessary for the production of certain proteins or cell surface markers. Cells that contain one or a few known human chromosomes in addition to the full complement of Chinese hamster chromosomes are being sought. If the difference between human and Chinese hamster chromosomes that results in differences in the degree of antibody binding to chromosomes from these two species is maintained in these hybrids, the antibody could be useful in screening hybridization products for human chromosomes. Coupling of antibody labeling to measurements of DNA content could allow the identification of the human chromosomes present in hybrid cells.

SECTION IV

STUDIES OF THE NUCLEOTIDE SEQUENCE IN NUCLEI AND CHROMOSOMES

Introduction

The ultimate aim in the study of nuclei or chromosomes is to establish the exact sequence of the base pairs in the DNA helix. To understand the genome and its expression, it is important to be able to detect and locate DNA stretches containing specific nucleotide base pair sequences. In the previous chapters, the total DNA, the average base composition, and the structural organization of chromatin in nuclei and chromosomes has been examined. The remaining chapters in this thesis present the results of experiments that aim at the detection and localization of specific DNA sequences in nuclei and chromosomes. The results in this section show that cross-linking the protein structures of chromosome and nuclei stabilizes these particles to such a degree that they remain intact, even when the DNA helix is separated into single strands. The presence of BrdU, a thymidine analog, is quantified using immunofluorescent labeling. In this way, the presence of single-stranded DNA can be demonstrated in chromosomes exposed to denaturing conditions while in suspension. These results make it possible to label specific DNA sequences in nuclei using in situ hybridization techniques. Flow cytometry is then used to detect and quantify these specific DNA sequences in single nuclei.

In situ hybridization is a relatively new technique that allows the chromosome sites of specific DNA sequences to be identified (figure IV.1) (Pardue and Gall, 1970; John et al., 1969; reviewed by Jones, 1973; Bauman et al., 1984). The DNA double helix in chromosomes fixed to slides is separated into single strands by incubating the slides at high pH. In the original procedure, the slides were incubated with radioactively labeled DNA sequence probes under conditions of high salt and high temperatures. These conditions facilitate annealing, or hybridizing, of the probes with complementary sequences in the chromosomes to reform a double-stranded helix. From the locations of silver grains after autoradiography, the sequences can be assigned to their chromosomal position. With in situ hybridization, it has been possible to localize single copy cellular genes relative to the bands in chromosomes. (Gerhard et al., 1981; Harper and

Saunders, 1981, Harper et al., 1981). Viral sequences have also been detected in infected cells using this technique (Brahic and Haase, 1978).

Recently, fluorescent or enzyme labels have been introduced to replace radioactive labels to visualize the target sequences in chromosomes on slides after in situ hybridization. These labels permit more accurate localization of chromosomal position and give immediate results. In situ hybridization procedures that employ fluorescent labels are of utmost importance in the adaptation of sequence detection for flow cytometric quantification. Several means are available to label hybridized probes fluorescently. RNA sequence probes can be labeled directly with fluorescent molecules (Bauman et al., 1980, 1981a, b, c). Alternatively, antibodies that recognize RNA-DNA hybrids can be used to localize hybridized RNA probes bound to DNA in chromosomes. A second antibody layer consisting of an anti-immunoglobulin conjugated to fluorescent molecules completes the visualization procedure (Rudkin and Stollar, 1977; van Prooijen-Knegt et al., 1982). A third means of sequence detection is shown

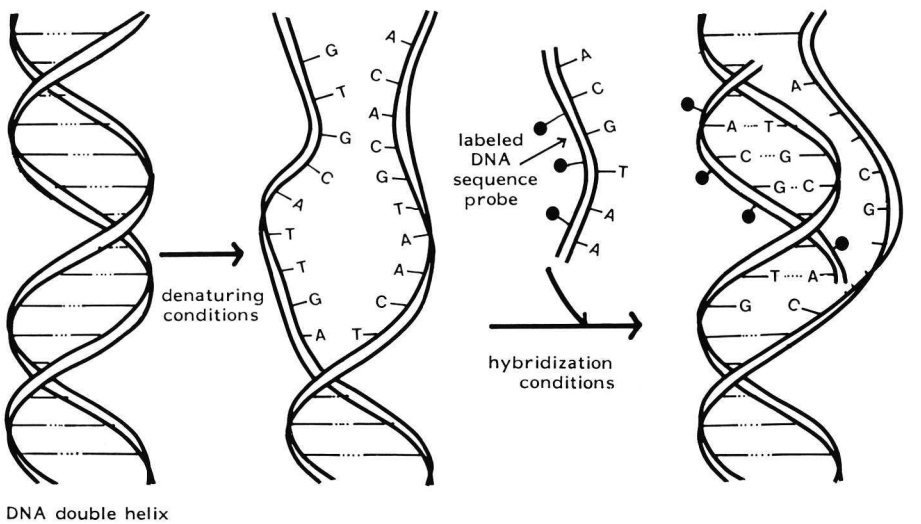


Figure IV.1. Schematic illustration of DNA helix denaturation and the hybridization of labeled DNA sequence probes. Under conditions of high pH or high temperature, strand separation occurs in the DNA. If a labeled DNA sequence probe is incubated under the proper conditions, it will compete with the complementary strand. It will anneal at points in the DNA helix where it can form adenine-thymidine (A-T) or guanine-cytosine (G-C) base pairs, or in other words, where the nucleotides it contains are complementary to those in the DNA. If the probe is labeled with ^{32}P, its presence in the DNA can be detected after autoradiography. If the probe is labeled with a molecule against which a specific antibody is available, the hybridized probe can be detected using non-autoradiographic means as illustrated in figure IV.2.

in figure IV.2. This procedure involves labeling the DNA sequence probes with a hapten against which an antibody with high specificity has been raised (for example, 2-acetylaminofluorene (AAF) (Tchen et al., 1984; Landegent et al., 1984) or biotin (Langer et al., 1981)). The hapten-labeled probe is allowed to hybridize to chromosomes fixed on slides. After hybridization, the bound probe is visualized in an indirect immunofluorescence procedure. the slides are incubated with an anti-hapten antibody, followed by incubation in anti-immunoglobulin conjugated to fluorescent molecules. The presence of the hybridized probe is detected by the fluorescent signal at the chromosomal location.

Although the chromosomal position of specific DNA sequences can be determined using in situ hybridization on slides, it is difficult to quantitate the extent or number of copies of the detected sequence in the genome with this technique. Furthermore, this technique is limited at present in its ability to reliably determine the frequency of cells

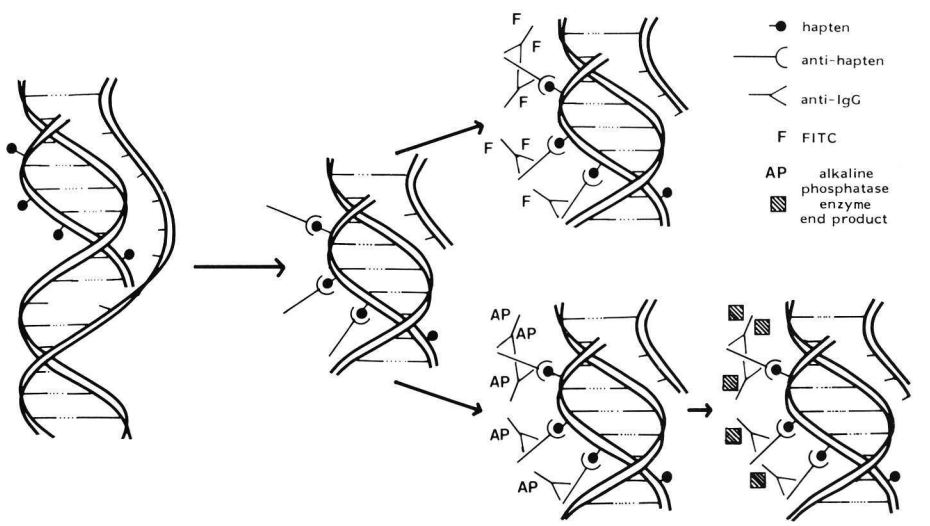


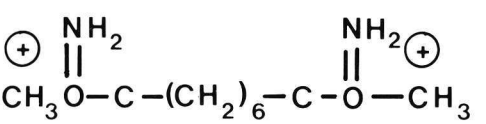
Figure IV.2. The detection of target DNA sequences using fluorescent or enzymatic labeling. In both cases, small antigenic molecules (haptens) are bound to the hybridized probe. After hybridization, the haptens are recognized by specific anti-hapten antibodies. After washing, a second antibody layer is added, which binds specifically to the anti-hapten molecules. This can be an anti-immunoglobulin conjugated to a fluorescent molecule, such as FITC. Alternatively, it can be an anti-immunoglobulin conjugated to an enzyme, such as alkaline phosphatase or horseradish peroxidase. In the first case, the bound probe is detected by the fluorescent signals. In the second case, deposits of dark crystals of enzyme end-product are formed at the hybridization sites after incubation in enzyme substrate.

containing a specific sequence. This is a result of the low number of cells affixed to slides and the time involved in noting the fluorescence signal for each cell.

Flow cytometry offers the means to overcome these limitations. Although topical resolution is lost in most flow cytometers, this loss is offered in exchange for the ability to quantify the specific fluorescence signal resulting from hybridized probe. Since single cells or chromosomes are rapidly analyzed, this tool has the potential to detect the frequency of particles that contain DNA sequences of interest within a larger heterogeneous population. If chromosomes can be labeled in suspension using in situ hybridization procedures, it may be possible to identify the chromosomes (using two DNA dyes), as well as the chromosomes to which specific DNA sequence probes bind. This could provide a rapid means of detecting translocated chromosomes.

The available in situ hybridization procedures have been restricted to cells or cell fragments that are fixed to a rigid support (e.g., a microscope slide or a filter). High temperatures and buffers that contain chelating agents and high concentrations of salt are required to denature the DNA helix and to efficiently and specifically hybridize DNA probes to homologous sequences. Under these conditions, nuclei and chromosomes in suspension disintegrate (chapter 13, 14). The stabilization of chromosome morphology and DNA content with Mg^{++} ions as described in section II is insufficient in the face of the rigorous conditions required for denaturation and hybridization.

In chapter 13, a means is shown to stabilize nuclei and chromosomes to overcome this problem. If the protein structure of chromatin is cross-linked with dimethylsuberimidate (DMS) (figure IV.3) chromosomes and nuclei can be denatured in suspension without loss of integrity. This integrity is demonstrated using flow cytometric measurements of DNA content. In a previous chapter (chapter 11), the stabilization of chromosomes with DMS was also shown to permit the fixation and staining on slides with a retention of morphology and banding pattern.



dimethylsuberimidate

Figure IV.3. Chemical formula of the protein cross-linker, dimethylsuberimidate (DMS).

To demonstrate that the DNA in DMS-treated chromosomes is indeed denatured, an anti-bromodeoxyuridine (anti-BrdU) antibody was employed. BrdU is incorporated in place of thymidine during DNA synthesis, if cells are grown in BrdU-containing medium. A monoclonal antibody specific for this molecule has been recently described (Gratzner, 1982). It recognizes BrdU only in single stranded DNA. Therefore, chromosomes or nuclei must be exposed to conditions that denature DNA if this antibody is to bind to BrdU. Anti-BrdU has been used with success to visualize sister chromatid exchanges in chromosomes fixed to slides (Gratzner et al., 1975; Pinkel, personal communication). It has also revolutionized the analysis of the cell cycle status and proliferative activity of cell populations (Dolbeare et al., 1983). Figure IV.4 shows the fluorescent distribution of nuclei from cells exposed to BrdU for a short period. After denaturation the nuclei can be labeled immunofluorescently with anti-BrdU and with a DNA-specific dye. The fluorescence intensities of both stains are

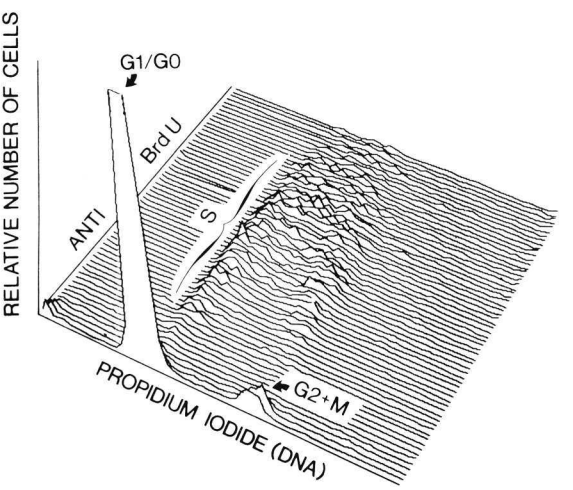


Figure IV.4. Bivariate fluorescence distribution of Chinese hamster cells, which were grown in bromodeoxyuridine (BrdU) for approximately 10 min (figure compliments of Dolbeare and Gray, Livermore). The cells were harvested, fixed, denatured and labeled with an anti-BrdU antibody. The cells were then incubated in anti-immunoglobulin-FITC and stained for DNA content with propidium iodide (PI). In a flow cytometer, the FITC and PI fluorescence of each cell in the population were analyzed (see text). Cells in S-phase actively synthesize DNA and incorporate BrdU rapidly. These cells have high green fluorescence and intermediate DNA content. Mid-S-phase cells take up BrdU more rapidly than early or late S-phase cells. Cells in G_1, G_0, G_2, or M are not synthesizing DNA and show no anti-BrdU fluorescence. With this type of analysis, the relative number of cells in S phase can be accurately determined. Compare this figure with figure 1.7.

quantified in a flow cytometer. The nuclei in S-phase show high anti-BrdU fluorescence indicating that DNA was rapidly synthesized. Cells in G_1 or G_2 have incorporated no BrdU and show no immunofluorescence.

The results in chapter 13 show that chromosomes containing BrdU can also be investigated with this antibody. A flow cytometer was used to quantify the binding of anti-BrdU to chromosomes. This study shows that the conformational state of DNA (single- versus double-stranded) after various denaturation treatments can be studied in single chromosomes using flow cytometry. The observations also imply that flow cytometry may be used to quantify other modified bases in chromosomes, if suitable immunological reagents are available.

DMS treatment and denaturation also affect the staining properties of nuclei with the DNA stain, propidium iodide. These effects are presented in chapter 13.

The results of successful in situ hybridization to nuclei in suspension are described in chapter 14. Large particles of chromatin, nuclei, and probes complementary to a large portion of the genome were chosen to demonstrate the feasability of in situ hybridization in suspension. Mouse thymocyte nuclei, stabilized with DMS, were hybridized to probes for mouse satellite DNA sequences. Mouse cell nuclei contain approximately 6 pg of DNA. Mouse satellite sequences comprise approximately 10% of the total genome in these cells. Mouse satellite sequences have been previously hybridized to chromosomes and nuclei on slides using several labeling procedures (Pardue and Gall, 1970, Jones and Robertson, 1970; Jones, 1970; Manuelidis et al., 1982; Landegent et al., 1984). The function of these sequences are not entirely known, but they may function in the germ line in some way (Singer, 1982).

In the procedure for in situ hybridization in suspension outlined in chapter 14, DMS-treated nuclei are denatured and hybridized overnight with mouse satellite DNA probes labelled with the AAF method (Landegent et al., 1984). Techniques were developed to increase nuclei recovery during the subsequent washing procedures and immunofluorescent labeling. These techniques allow hybridization to be performed on as few as 10^4 nuclei. Dual beam flow cytometry is employed to quantify the amount of hybridized probe, as well as the DNA content of each nucleus. The results in this chapter show that the specific fluorescent signal of mouse nuclei hybridized to satellite DNA probes is on average 20-fold higher than the signals measured for nuclei hybridized to a heterologous sequence probe.

Hybridization labeling procedures can also be used to identify specific DNA sequences in DNA affixed to nitrocellulose filters. DNA from cells or from fractionated chromosomes can be denatured and fixed to these filters. The filter is incubated under hybridization conditions with

labeled DNA sequence probes. After stringent washing, the target sequences can be visualized using autoradiography, in the case of radiolabeled probes, or with local deposits of enzyme end-products, in the case of probes labeled using an immunoenzymatic procedure (figure IV.2). In chapter 15, filter hybridization is employed to detect the presence of specific nucleotide sequences in single nuclei and chromosomes. Using an immuno-enzymatic protocol, target sequences are visualized with a sensitivity of less than 0.6 pg DNA.

The experiments in this section illustrate that flow cytometry can be used to detect and quantify the stretches of DNA with a specific nucleotide sequence in nuclei and chromosomes. The specificity of in situ hybridization techniques can be combined with the ability to quantify the specific signal with flow cytometry. Since single nuclei are visualized both in suspension and on filters, these techniques may be a means to determine the frequency of cells containing a specific sequence in a population.

Chapter 13

STABILIZATION OF CHROMOSOMES TO WITHSTAND DENATURATION IN SUSPENSION AND QUANTIFICATION OF SINGLE-STRANDED DNA WITH FLOW CYTOMETRY

SUMMARY

A procedure is described for the stabilizaton of chromosomes in suspension that permits chromosomes to be incubated under conditions used to denature the DNA helix. The degree of single strand formation can be determined with flow cytometry after an immunofluorescent labelling procedure. Isolated chromsomes treated with the protein cross-linker, dimethylsuberimidate (DMS), are stable to a variety of denaturation and hybridization conditions. The integrity of chromosomes is demonstrated by a well-resolved fluorescence distribution after DNA staining, as measured in a flow cytometer, and the preservation of normal chromosome morphology, as observed with a light microscope. Without DMS treatment, the chromosomes fall apart rapidly. The ability of a monoclonal antibody that recognizes bromodeoxyuridine (BrdU) only in single-stranded DNA to bind to BrdU-containing chromosomes is quantified in a flow cytometer. In this way, the relative degree of strand separation in DMS-treated chromosomes incubated under various conditions can be determined.

INTRODUCTION

The biochemical properties of chromosomes in suspension can be analyzed quantitatively with flow cytometry (Gray et al., 1979b; chapter 7, 12). The information that can be derived from the measured optical signals depends on the fluorescent labeling procedures that can be employed. Since the integrity of the chromosomes in suspension depends on the integrity of the DNA helix, labelling procedures that require strand separation cannot be applied directly. Studies of the nucleotide sequence in the DNA of chromosomes with base-specific antibodies or with DNA or RNA sequence specific probes, however, require strand separation. This paper describes a technique for stabilizing the protein structure of chromosomes such that the DNA strands can be separated without affecting the integrity of the particles in suspension. This is done by cross-linking the proteins with

dimethylsuberimidate (DMS). DMS is a cross-linking agent that reacts at high pH to form 10-nm bridges between protein molecules (Peters and Richards, 1977). This reagent has been used to study the conformational arrangement of enzyme subunits and the components of erythrocyte membranes (Peters and Richardson, 1977). The structural organization of chromosomes can also be preserved with DMS treatment to allow isolated chromosomes to be centrifuged onto microscope slides and banded for identification (chapter 11).

Strand separation in chromosomes is demonstrated using an monoclonal antibody against bromodeoxyuridine (BrdU) (Gratzner, 1982). If cells are grown in its presence, BrdU is incorporated in place of thymidine during DNA synthesis. The anti-BrdU antibody has been used to demonstrate sister chromatid exchange on slides (Gratzner et al., 1975) and to study DNA synthesis and proliferative activity in cells (Dolbeare et al., 1983). The antibody recognizes BrdU only in single-stranded DNA and requires the denaturation of chromatin to bind (Dolbeare et al., 1983). This property makes this anti-BrdU antibody an ideal immunological reagent for assaying single strand formation in chromosomes.

In this chapter, results are presented to show that the conformational state of DNA in individual chromosomes can be assayed using flow cytometry and an indirect immunofluorescence procedure employing the anti-BrdU antibody. The potential of these results for combining in situ hybridization in chromosomes and flow cytometric quantification are discussed.

MATERIALS AND METHODS

Chromosome and nuclei preparation

Chromosomes were prepared from a Chinese hamster fibroblast culture derived from the M3-1 cell line. The cells were maintained in exponential growth as described in chapter 6. Chromosomes were isolated from mitotic cells using the isolation procedure described in chapter 6. For some experiments, nuclei were isolated from the thymocytes of 6-8 wk old BC3 female mice using the isolation procedure described in chapter 8.

Bromodeoxyuridine labeling

Where indicated, bromodeoxyuridine (BrdU, Sigma) was added to the culture medium of fibroblast cell lines 12 h before cell collection to a final concentration of 10 μM.

Dimethylsuberimidate (DMS) treatment

For DMS treatment, K_2CO_3 and DMS were added to a suspension of $5x10^6$ nuclei/ml or approximately $5x10^6$ chromosomes/ml from a concentrated stock solution mixed immediately before use. The final concentrations were 20 mM and 3 mM, respectively. The resultant pH was 10. After 15 min at 25°C, the pH was adjusted to 8.0 by the addition of 50 µl 100 mM citric acid/ml. In some experiments, this process was repeated.

Denaturation and incubation in hybridization buffers

Salt or formamide were added to chromosome suspensions at concentrations indicated. SSC (Standard Saline Citrate) was added from a 20 times concentrated stock solution. 1xSSC is equivalent to 0.15 M NaCl plus 0.015 M Na-citrate. Formamide was added to final concentration of 50% (v/v) from a 100% solution (Kodak). The chromosomes were incubated at the temperatures indicated (where not specificed 1 h, 25 °C) and diluted in isolation buffer containing $MgSO_4$ (IB+M, containing 50 mM KCL, 5 mM HEPES, and 10 mM $MgSO_4$) before being stained. Chromosome suspensions were incubated at the indicated temperatures in water bath.

Immunofluorescent labeling with anti-BrdU

Chromosomes were isolated from cells grown either in the presence or absence of BrdU as described above, with the exception that the reducing agent, dithiothreitol, was omitted from isolation buffer. The chromosomes were treated with DMS. Samples containing 0.5 ml chromosome suspension were used. In some experiments, formamide was added to 50% final concentration. After the chromosome suspensions were incubated in hot water baths at temperatures indicated in results, the tubes were placed quickly on ice. Formamide-containing suspensions were diluted by adding an equal volume of cold IB+M. Anti-BrdU (IU-1, a gift from M. Vanderlaan and F. Dolbeare, Livermore) and FCS were added to 1:10 dilution and 2% final concentration, respectively. The suspensions were incubated 15 min at room temperature and subsequently centrifuged 15 min at 300g. After removal of the supernatant fluid, the pellet was resuspended in 0.5 ml IB+M containing 2% FCS, 0.25% Triton X-100, and a 1:10 dilution of anti-mouse immunoglobulin conjugated to fluorescein molecules (FITC) (Nordic, Tilburg, The Netherlands). After incubation at room temperature for 15 min, the suspension was layered carefully on 1 ml IB+M containing 10% FCS and centrifuged 10 min at 300g. The pellet was resuspended in 1.0 ml IB+M.

DNA staining

Chromosome and nuclei suspensions were syringed repeatedly through a 23-gauge needle using a 1- or 3-ml syringe, filtered through 50 μm nylon mesh and stained for DNA content using either 2.7 μM Hoechst 33258 (HO) or 27 μM propidium iodide (PI).

Flow cytometric analysis

Chromosome and nuclei suspensions were analyzed using the LLL dual beam cytometer (Dean and Pinkel, 1978) as described in chapter 12. For figures 13.1 and 13.2, the amplifier gains were adjusted for the measurement of each panel to utilize the full 256 channels of the pulse height analyzer. The FITC fluorescence was measured using a logarithmic amplifier in which 20 channels is equivalent to approximately 5 to 10-fold difference in fluorescence intensity.

RESULTS

Effect of DMS treatment on the DNA distributions of chromosomes

The effect of the protein cross-linker dimethylsuberimidate (DMS) on the resolution of chromosomes in a DNA fluorescence distribution was determined. Figure 13.1 shows that the fluorescence distributions of Chinese hamster chromosomes with and without DMS treatment are very

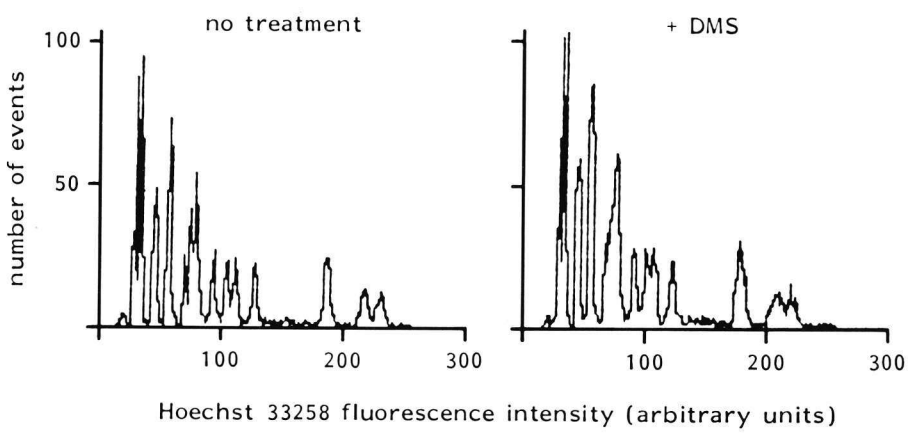


Figure 13.1. The effect of the protein cross-linker, dimethylsuberimidate (DMS) on the fluorescence distribution of Chinese hamster chromosomes measured in a flow cytometer after staining with Hoechst 33258. Left panel (A): no DMS treatment. Right panel (B): treated with DMS.

similar. The chromosomes were stained with Hoechst 33258 (HO) and measured in flow cytometer. It is possible to recognize virtually all of the chromosomes of this cell line in both panels.

Effect of DMS treatment on chromosome stability

After DMS treatment, chromosomes in suspension can withstand conditions which denature the DNA helix. The effect of media and temperatures that are commonly used to induce strand separation for DNA-DNA hybridization experiments (Jones, 1973) was determined. These protocols

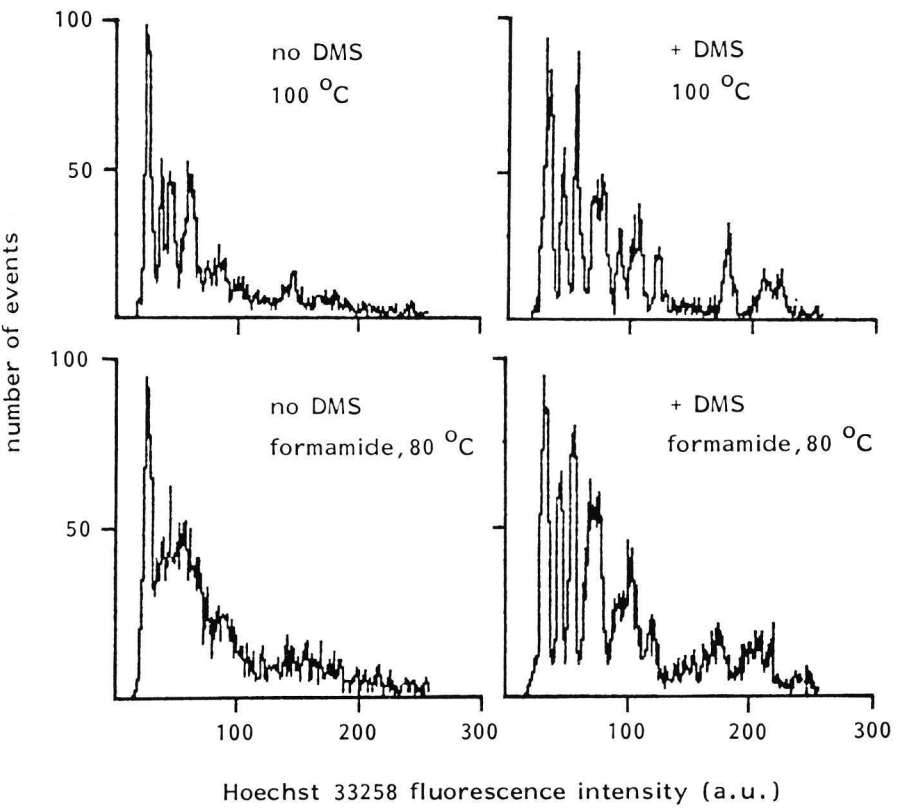


Figure 13.2. The effect of denaturing conditions on chromosomes from Chinese hamster culture cells. Chromosomes were treated with DMS before denaturation for the measurements in the right panels. Chromosomes were incubated for 15 min at 100°C in the top panels. For the bottom panels, chromosomes were incubated in 50% formamide at 80°C for 15 min and then were diluted with isolation buffer. The chromosomes were stained with HO before measurement of the fluorescence distributions.

make use of buffers containing high salt and chelating agent concentrations (SSC) (Landegent et al., 1984) and formamide (McConaughy et al., 1969) followed by incubation at high temperatures. Chromosomes were subjected to several of these procedures, and, after cooling, the chromosomes were stained with HO. A fluorescence distribution was measured for each suspension in a flow cytometer. Figure 13.2 shows a typical experiment. The DNA distribution of untreated chromosomes shows significantly fewer resolved chromosomes and a higher debris level than that of DMS-treated chromosomes.

The effect of DMS treatment on the stability of chromosomes under a series of conditions as determined from the quality of the measured DNA distribution is summarized in table 13.1. The results presented here indicate that DMS-treated chromosomes are stable under more harsh conditions than untreated chromosomes. Under some conditions, two repeated treatments with DMS are more effective for chromosome stabilization than a single treatment.

The optimal concentration of DMS for chromosome stabilization is 10 mM. Lower concentrations are insufficient in stabilizing the chromosomes.

Table 13.1

EFFECT OF DMS TREATMENT ON THE ABILITY OF CHROMOSOMES TO WITHSTAND VARIOUS HYBRIDIZATION BUFFERS AND DENATURATION CONDITIONS AS MEASURED IN A FLOW CYTOMETER*

additions to medium and temperature	times treated with 3 mM dimethylsuberimidate		
	0	1	2
none	++	++	++
2xSSC	+	+	+
100°C, 10 min	-	+	+
2xSSC, 100°C, 10 min	--	(--)	+
50% formamide	(-)	(+)	(+)
50% formamide, 2xSSC	-	(-)	+
50% formamide, 70°C, 10 min	--	-	+
50% formamide, 2xSSC, 70°C, 10 min	--	(--)	-

*The resolution of peaks in fluorescence distributions of PI-stained Chinese hamster chromosomes were judged: ++ excellent, + good, - poor, -- very poor. Note: judgements in parentheses ( ) are based on chromosome morphology as observed with light microscope.

Effect of salt and high incubation temperatures on DMS-treated chromosomes

The results in table 13.1 indicate that DMS-treated chromosomes maintain their integrity under several conditions used for DNA denaturation. Chromosomes treated with DMS one time are not, however, maintained under conditions that combine high salt and chelating agent concentrations with high temperatures or with formamide. This was further investigated by examining the morphology of DMS-treated chromosomes with a fluorescence microscope after their incubation at several different temperatures in various SSC concentrations (table 13.2). As the salt and chelating agent concentrations are increased by the addition of 0 to 3xSSC, the temperature decreases at which chromosomes fall apart. These chromosomes swell substantially in 2-3xSSC at low temperatures, but remain intact. At higher temperatures, the chromosomes fall apart. In contrast, chromosomes treated twice with DMS can withstand the combination of 2xSSC and boiling.

Table 13.2

EFFECT OF SSC* CONCENTRATION AND INCUBATION TEMPERATURES ON THE MORPHOLOGICAL INTEGRITY OF ISOLATED CHROMOSOMES TREATED ONCE WITH DIMETHYLSUBERIMIDATE (DMS)**

temperature	0xSSC	1xSSC	2xSSC	3xSSC
25°C, 2 h	++	++	+/-	+/-
36°C, 2 h	++	++	++	++
60°C, 2 h	++	++	+/-	--
80°C, 2 h	++	++	+/-	--
100°C, 3 min	++	++	--(++)	--
100°C, 10 min	++	+/-	--(++)	--

++, chromosomes intact or only slightly swollen.
+/-, chromosomes very swollen.
--, chromosomes fallen apart into mass of chromatin, not recognizable as chromosome.

* 1xSSC = 0.15 M NaCl, 0.015 M Na-citrate.
**Morphology of chromosomes treated not once, but two repeated times, with DMS before treatment are indicated in parentheses ( ).

Chromosomes were isolated, treated with 3 mM DMS after isolation. The additions indicated were added to chromosomes in isolation buffer. The chromosomes were incubated at the indicated temperatures and, after 2 h, were stained with Hoechst 33258 (2.7 µM). Chromosome morphology was judged by observation under fluorescent microscope with UV excitation.

Anti-BrdU labeling

An immunofluorescence labeling procedure and flow cytometry were employed to determine the extent of single strand formation in DMS-treated chromosomes exposed to conditions for DNA denaturation. Chromosomes were isolated from cells grown 12 h in the presence or absence of bromodeoxyuridine (BrdU). The chromosomes were stabilized with DMS. The chromosome suspensions were denatured by boiling for 15 min and were quenched quickly on ice to minimize renaturation of the DNA helix. Chromosomes were labeled with a monoclonal antibody that recognizes BrdU only in single stranded DNA, followed by incubation with an anti-mouse immunoglobulin-FITC antibody. The DNA in the chromosomes was stained with PI. Figure 13.3 shows that the FITC fluorescence intensity of BrdU-containing chromosomes (right panel) is significantly higher (approximately 10 times) than the fluorescence of chromosomes from cells not grown in BrdU.

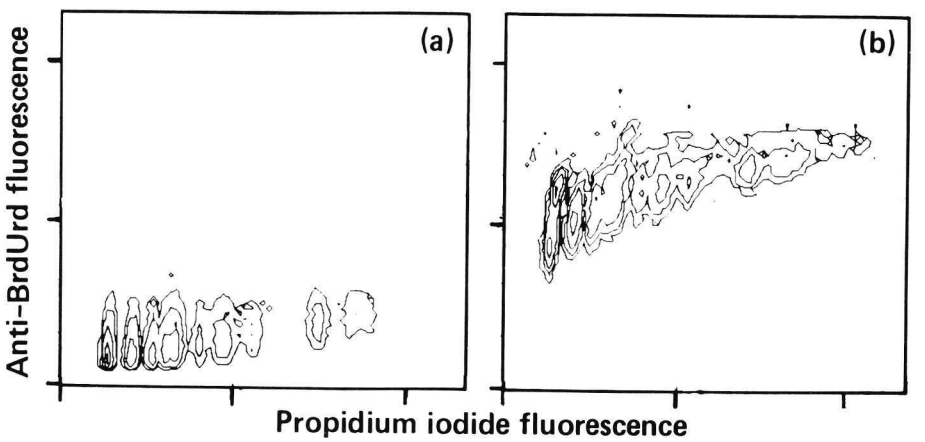


Figure 13.3. Bivariate distributions of FITC vs. propidium iodide (PI) fluorescence of Chinese hamster chromosomes. The cells used for the left panel (A) were grown in the absence of bromodeoxyuridine (BrdU). The cells used for the right panel were grown 12 h in the presence of BrdU. In both cases, the chromosomes were isolated, stabilized by DMS treatment, and denatured by 15 min incubation at 100°C. The chromosomes were then labeled in an indirect immunofluorescent procedure with an antibody specific for BrdU in single-stranded DNA, followed by an anti-immunoglobulin conjugated to FITC. The chromosomes were then stained with the DNA-fluorochrome, PI. The chromosomes were analyzed in a dual beam flow cytometer. The FITC fluorescence of each chromosome is plotted along the ordinate on a logarithmic scale (20 channels represents approximately a 5 to 10-fold difference in fluorescence intensity). The PI fluorescence is plotted along the abscissa using a linear scale.

DNA resolution after denaturation

The resolution of chromosomes in a DNA fluorescence distribution is well preserved despite DMS treatment, denaturation, and immunofluorescent labeling. The events in the right panel of figure 13.3 were compressed onto the DNA fluorescence axis and are displayed in figure 13.4. Although the fluorescence intensities are expressed in 64 channels, rather than in 256 channels as was the case in figures 13.1 and 2, the various chromosomes from Chinese hamster cells can be identified in this distribution.

Assay of single-strand formation

Anti-BrdU labeling can be used to compare the degree of single-strand formation in chromosomes incubated under various denaturation conditions (figure 13.5). The FITC fluorescence intensity of chromosome 2 was determined from bivariate fluorescence distributions like those shown in figure 13.3. This chromosome can be easily recognized in the distributions by its DNA fluorescence intensity. The fluorescence intensity of BrdU-containing chromosomes is significantly higher if the chromosomes are boiled for 15 min, rather than 5 min, before immunofluorescent labeling.

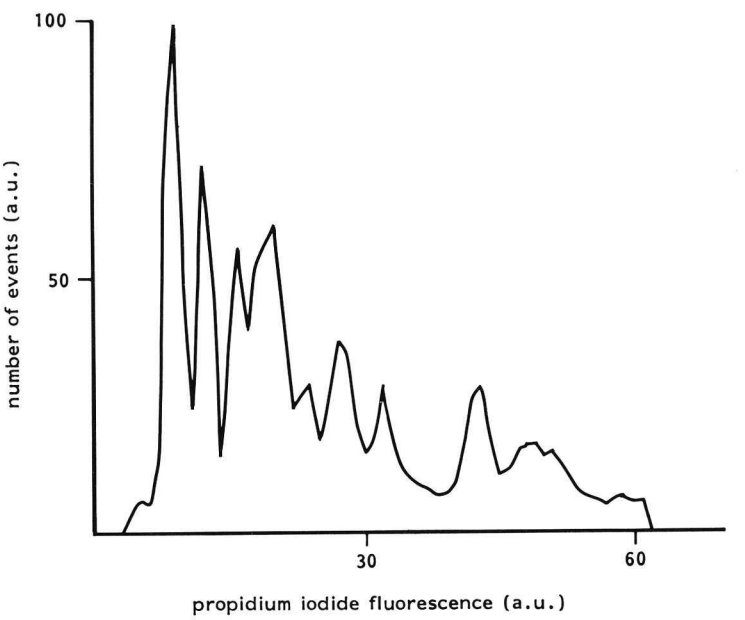


Figure 13.4. The PI fluorescence distribution of chromosomes measured in the right panel of figure 13.3. The data in figure 13.3 were compressed on the abscissa to obtain this representation. The abscissa is divided into only 64 channels (compared with 256 in figures 13.1 and 13.2).

Boiling decreases the level of the aspecific fluorescence measured for chromosomes that do not contain BrdU. The fluorescence of BrdU containing chromosomes after denaturation at 80°C in 50% formamide is as high as that of the chromosomes boiled in IB+M for 15 min. However, the aspecific fluorescence of chromosomes that do not contain BrdU is significantly higher in the presence of formamide. Similar results were observed in three repeated experiments, and also when HO was used as the DNA stain.

Effect of DMS and denaturation on absolute DNA fluorescence

Some shifts in the fluorescence intensity of PI-stained chromosomes with DMS treatment were noted in the experiments described above. The effect of DMS treatment treatment and denaturation on the absolute fluorescence intensity of chromatin stained with PI was investigated using

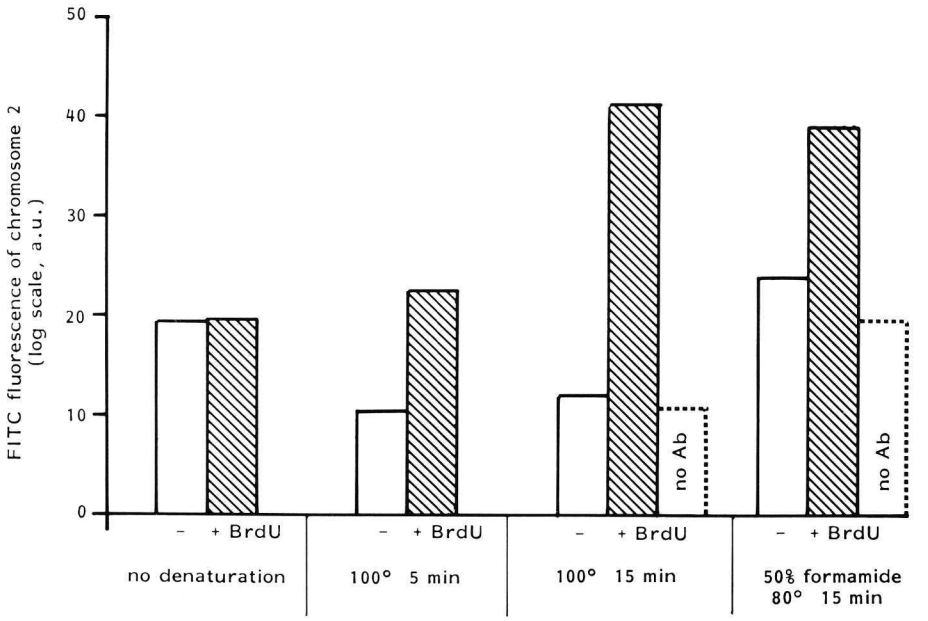


Figure 13.5. The FITC fluorescence intensity of the Chinese hamster chromosome 2 after various treatments. The fluorescence intensity is expressed on a logarithmic scale (20 channels represent a 5 to 10-fold difference in fluorescence intensity). Chromosomes were isolated from cells grown either in the presence (hatched bars) or absence (open bars) of BrdU. The chromosomes were stabilized with DMS and incubated under various conditions as indicated along the horizontal axis. The chromosomes were labeled in an indirect immunofluorescence procedure with an antibody specific for BrdU in single-stranded DNA. The dotted bars indicate the fluorescence of BrdU-containing chromosome 2 after the indicated denaturing conditions, but with no antibody labeling.

mouse thymocyte nuclei. Isolated nuclei have been shown to be a good model for fluorescent staining of chromosomes in suspension (chapter 8). The fluorescence distribution of nuclei forms a narrow peak. Furthermore, nuclei can be incubated in suspension at 100°C without DMS treatment, allowing studies of the effect of boiling on chromatin staining. Nuclei were subjected to DMS treatment and/or incubation at 100°C. Treated and untreated nuclei were mixed together and then stained with PI. In this way, the change in PI fluorescence caused by the treatment can be accurately determined.

The PI fluorescence intensity of nuclei cross-linked with DMS was approximately 40% lower than that of untreated nuclei. Boiling increased the PI fluorescence of untreated and DMS-treated nuclei 50% and 145%, respectively. After boiling, untreated and DMS-treated nuclei have the same PI fluorescence. These values represent the results of three repeated experiments.

DISCUSSION

The observations presented here indicate that an intact protein structure, as well as an intact DNA helix, are crucial for the maintenance of chromosome integrity in suspension. If measures are not taken, isolated chromosomes fall apart under conditions used to induce strand separation in the DNA helix. If the protein structure of the chromatin is cross-linked with DMS, however, the morphology of nuclei and chromosomes can be maintained under these conditions. DMS-treated chromosomes show well-resolved peaks in a DNA fluorescence distribution, indicating preserved integrity, even after denaturation and immunofluorescent labeling procedures.

The experiments with BrdU-labeled chromosomes demonstrate that strand separation is achieved in DMS-treated chromosomes. The amount of binding of an antibody specific for BrdU in single-stranded DNA is a measure of DNA denaturation. Antibody labeling was performed at a lower temperature than that used for DNA strand separation. At the time that the chromosomes are stained with propidium iodide, considerable renaturation has occurred. PI intercalates into the DNA helix and requires double-stranded DNA (LePecq and Paoletti, 1967). Despite this, significant levels of anti-BrdU labelling, and thus single DNA strands, were detected in the chromosomes. The results also show that it is possible to use anti-BrdU labeling to compare the amount of single strands formed after the chromosomes have been subjected to various conditions. The data show, for example, that significantly more single strands are formed in chromosomes after 15 min incubation at 100°C than after 5 min incubation.

In this study, some differences were detected among Chinese hamster chromosomes in the extent of anti-BrdU labeling. Populations of chromosomes are visible in the right panel of figure 13.3 that show higher anti-BrdU fluorescence than would be expected from their total DNA content. This may reflect a higher adenine-thymidine to guanine-cytosine ratio in these chromosomes and, therefore, a higher level BrdU incorporation. Alternatively, it may indicate that structural organization and the susceptibility to denaturation vary among Chinese hamster chromosomes.

Although a single DMS treatment maintains the morphology of chromosomes under many conditions that result in DNA denaturation, the chromosomes are instable under conditions which combine high SSC concentrations with high temperature. SSC is a solution of NaCl and the chelating agent, sodium citrate. The effect of SSC on chromosomes is somewhat paradoxical in that it is used in hybridization experiments to stabilize the DNA double helix through shielding the negatively charged phosphate groups (Cole et al., 1972). It is also used to inhibit nucleases, which require divalent cations for activity. The effect of SSC on chromosomes may reflect the importance of proteins for chromosome morphology when the DNA is denatured in suspension. High salt concentrations and boiling temperatures are known to remove proteins from chromatin (Nicolini et al., 1975; Bolund and Johns, 1973). It is possible that, despite DMS-treatment, crucial structural proteins are removed under these conditions. The ability of chromosomes cross-linked with two repeated DMS treatments to withstand high SSC concentrations at high temperatures may reflect a higher degree of cross-linking and better protein retention in these chromosomes. Alternatively, the sodium citrate in SSC may remove Mg^{++} ions, and in so doing, cause chromosome disruption. Mg^{++} ions are known to stabilize the DNA helix (Eichhorn, 1962; Tabor, 1962; Baba and Kagemoto, 1974), albeit at low ionic strengths (Zimmer et al., 1971), and to maintain chromatin in a condensed state (Aaronson and Woo, 1981, Olins and Olins, 1972).

There is some evidence that DMS limits the accessibility of PI to chromatin. The PI fluorescence intensity of DMS-treated nuclei is lower than that of untreated nuclei. Proteins are also known to decrease the binding of intercalating dyes such as PI to chromosomes (Williams et al., 1972; Angerer and Moudrianakis, 1972; Langlois et al., 1980; Brodie et al., 1975) and DMS may interfere with PI binding by similar mechanisms. These molecules may mask available sites in chromatin (Angerer and Moudrianakis, 1972), or may stabilize the helix to prevent the conformational changes (Waring, 1970) that contribute to cooperative binding of PI (Angerer and Moudrianikis, 1972). Preliminary experiments indicate that staining by Hoechst 33258, a nonintercalating dye, is unaffected by DMS treatment.

Both DMS- and untreated nuclei show large increases in PI fluorescence after boiling. Boiling also reduced the level of aspecific anti-BrdU binding observed in DMS-treated chromosomes. These observations may point to the loss of proteins or DMS molecules, which may associate aspecifically with antibody molecules as well as interfere with PI binding. The PI fluorescence of nuclei has been shown previously to increase substantially after removal of proteins by acid treatment (Darzynkiewicz et al., 1984).

The data show that flow cytometry can be used in combination with DNA and immunofluorescent staining to study the conformation of DNA and the degree of single strand formation. In addition, specific nucleotides in the DNA of individual chromosomes can be quantified using immunological reagents and flow cytometry. These observations may have applications in studies now performed on chromosomes fixed to microscope slides. It may be possible to quantify in single chromosomes the frequency of modified bases in DNA (Baan et al., 1982) or organizational changes in DNA produced, for example, by UV irradiation (Schreck et al., 1974). The conformational status of the DNA of fixed cells and nuclei has also been studied using flow cytometry and acridine orange staining (Darzynkiewicz et al., 1974, 1975). It may also be possible to locate and quantify the extent of areas in chromosomes that rapidly reassociate after denaturation (Mace et al., 1972). It is known that some regions of the genome replicate earlier than others (Cremer and Gray, 1983). With controlled administration of BrdU to dividing cells, it may be possible to identify the chromosomes containing these areas using anti-BrdU and flow cytometry.

This series of experiments shows that chromosomes can be stabilized in suspension with DMS buffers and temperatures used for DNA denaturation and hybridization. They can also be labeled using indirect immunofluorescent procedures. The fluorescence distribution shows sufficient resolution after these procedures for the identification of the chromosomes of Chinese hamster cells. These findings are the foundations for labeling specific DNA sequences in suspended chromosomes using in situ hybridization techniques.

Chapter 14

DETECTION OF DNA SEQUENCES IN NUCLEI BY IN SITU HYBRIDIZATION IN SUSPENSION AND DUAL-BEAM FLOW CYTOMETRY

Flow cytometry is widely used for the analysis of cell nuclei and chromosomes. After labeling with fluorescent dyes, the DNA content (Crissman and Tobey, 1974; Gray et al., 1979a, b; Darzynkiewicz et al., 1980; Dolbeare et al., 1983), base composition (Langlois et al., 1980), and structural proteins (Roti Roti et al., 1982; chapter 12) in chromatin can be rapidly quantified. The logical next application is the detection and quantification of specific DNA sequences using fluorescent DNA hybridization techniques. A report is made in this chapter on a method to stabilize nuclei in suspension so that their integrity can be maintained during denaturation and hybridization. To demonstrate that in situ hybridization can be performed in suspension, stabilized mouse thymocyte nuclei are hybridized with a probe for mouse satellite DNA sequences. A protocol using 2-acetylamino-fluorene (AAF)-modified DNA probes and indirect immunofluorescence (Landegent et al., 1984) is employed to visualize the target sequences. With dual beam flow cytometry (Dean and Pinkel, 1978), both the amount of hybridized probe and the DNA content of individual nuclei are determined. These results demonstrate that the specificity of DNA hybridization can be combined with the speed and quantitative analysis provided by flow cytometry.

In a dual beam flow cytometer, cells or cell fragments, suspended in fluid, pass in a single file through two lasers, which are tuned to emit light at different wavelengths. Cells that have been labeled with fluorescent molecules excitable at these wavelengths will fluoresce. The fluorescence intensity from each passing cell is proportional to the number of bound molecules (Visser et al., 1978; Kerker et al., 1982). The fluorescence signals emitted by each cell in a suspension are detected, amplified and collected in a multi-channel histogram. The fluorescence emission signals from the two excitation beams can be quantified separately using a combination of spatial and chromatic filtering. Thus, the binding of fluorescent markers to individual cells in a larger, heterogeneous population can be determined.

Several laboratories have developed fluorescent labeling procedures that allow the visualization of hybridized DNA sequence probes in cells fixed to slides (Rudkin and Stollar, 1977; Bauman et al., 1980; van Prooijen-Knegt et al., 1982; Langer-Safer et al., 1982; Landegent et al., 1984). DNA hybridizations are usually performed in solutions that contain chelating agents and high concentrations of salt. Long incubations at high temperatures facilitate and ensure specific hybridization. Nuclei, isolated into suspension using a procedure developed for the isolation of chromosomes from mitotic cells (chapters 6 and 7), fall apart rapidly under these conditions. The results in table 14.1 demonstrate that the nuclei can be stabilized by cross-linking the nuclear proteins with dimethylsuberimidate (DMS). Nuclei that have been treated with DMS can withstand a variety of denaturation and hybridization conditions. DMS-treated nuclei maintain their morphology in suspension. After DNA staining with Hoechst 33258, these nuclei show normal fluorescence distributions consisting only of a narrow peak when they are measured in a flow cytometer. Without DMS treatment, the peak is broad or is replaced by a debris continuum.

To demonstrate that nuclei pretreated in this way are still suitable for in situ hybridization procedures, DMS-treated mouse thymocyte nuclei were hybridized with a probe for mouse satellite DNA sequences. These

Table 14.1

EFFECT OF DIMETHYLSUBERIMIDATE (DMS) TREATMENT ON THE ABILITY OF NUCLEI TO WITHSTAND VARIOUS HYBRIDIZATION BUFFERS AND DENATURATION CONDITIONS

treatment	no DMS	DMS treated
none	++	++
100°C 10 min, + Mg^{++}	++	++
100°C 10 min, - Mg^{++}	-	++
2xSSC, + Mg^{++}	+	++
2xSSC, - Mg^{++}	--	++
2xSSC, 100°C 10 min, + Mg^{++}	+	++
2xSSC, 100°C 10 min, - Mg^{++}	--	++
50% formamide, 70°C, 10 min, + Mg^{++}	++	++
50% formamide, 2xSSC, +/- Mg^{++}	--	++
50% formamide, 2xSSC, 70°C, + Mg^{++}	--	+
0.07 NaOH, 25°C, 2 min	--	--

Quality of DNA distribution measured by flow cytometry:
++, excellent; +, good; -, poor; --, very poor.

sequences comprise approximately 10% of the total DNA in mouse nuclei (Waring and Britten, 1966). Total human DNA served as a control; this material shows no cross-hybridization to mouse DNA on filters or slides. The probes were fluorescently labeled with the AAF procedure (Landegent et al., 1984; Tchen et al., 1984). Approximately 20% of the guanine residues in the DNA probes were chemically modified with N-acetoxy-2-acetylamino-fluorene (N-AcO-AAF). Nuclei, suspended in hybridization buffer in the presence of AAF-labelled probe, were denatured and incubated at the hybridization temperature overnight. After hybridization, the bound probe was visualized with a rabbit anti-AAF antibody (Baan et al., 1982) and a goat-anti-rabbit immunoglobulin conjugated to rhodamine (TRITC).

The amount of mouse satellite probe required to hybridize with the number of nuclei routinely used in flow cytometry (10^6) is more than most DNA chemists want to part with on a routine basis (12 μg for hybridization in probe excess). For this reason, the procedure was scaled down to require only 10-20 thousand nuclei and a hybridization volume of approximately 50 μl. To reduce loss of nuclei during the washing procedures after hybridization, 10^7 mouse erythrocytes were added after hybridization. The erythrocytes were also cross-linked with DMS to prevent their disruption in the hypotonic buffers used in the immunological procedures. We have found that the added DMS-treated erythrocytes result in a large pellet, which contains the nuclei and can be handled conveniently.

Added erythrocytes do not interfere with the flow cytometric measurement of hybridized probe. In a dual beam cytometer, individual nuclei were identified at the first laser by the fluorescence of the DNA stain, Hoechst 33258. Erythrocytes and clumps of nuclei were then disregarded in the measurement of TRITC fluorescence at the second laser.

Fluorescent micrographs of nuclei that were hybridized in suspension and then centrifuged onto microscope slides, as described in the legend of figure 14.2, show that binding of the mouse satellite probe is specific and localized to the heterochromatic regions in the nuclei (figure 14.1). No binding of the human DNA probe could be observed. Suspensions of nuclei hybridized in the absence of either probe showed no red fluorescence after immunofluorescent labeling procedures.

Bivariate dot plots of the TRITC versus Hoechst fluorescence intensity of nuclei hybridized in suspension and measured in a dual-beam flow cytometer are shown in figure 14.2. Each dot represents the measurements of a single nucleus. From the cumulative frequency curves shown in figure 14.3, the median TRITC fluorescence intensity of the nuclei in each panel can be determined. The median of nuclei hybridized with mouse satellite DNA is approximately 20 times higher than that of nuclei hybridized with

the human DNA probe (average of 5 separate experiments, range: 17-31 times). This value is corrected for the background signals measured for nuclei receiving only antibody. The fluorescence intensity measured for nuclei hybridized with the human DNA probe is only slightly higher than that measured for nuclei hybridized in the absence of probe, with or without antibody treatment.

The procedure for hybridization in suspension differs from the protocol for slides (Landegent et al., 1984) in the reduced number of wash steps. The data show that this does not result in aspecific antibody binding or aspecific hybridization. As on slides, however, the stringency of the hybridization conditions is important for specific labeling in suspension. Other conditions tested gave less satisfactory results than

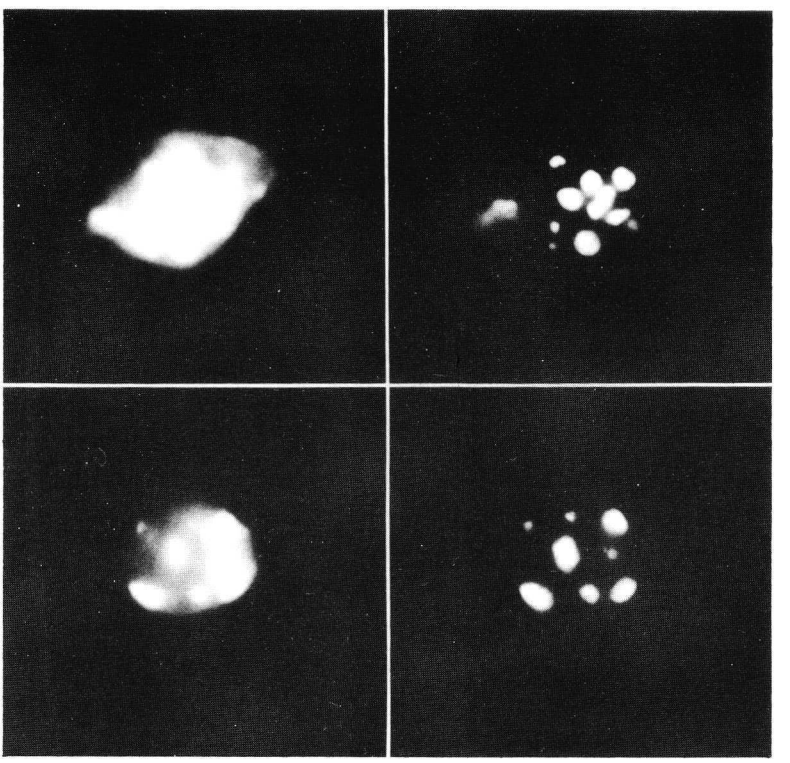


Figure 14.1. Hybridization of mouse satellite DNA mouse thymocyte nuclei in suspension. Photographs of two nuclei show the blue Hoechst 33258 fluorescence, illustrating the more intensely staining, A-T-rich heterochromatin areas in the nuclei (left panels), and the red rhodamine (TRITC) fluorescence, indicating bound probe (right panels). The actual size of the nuclei is approximately 5 µM.

the protocol presented here. For example, nuclei hybridized at 60°C without formamide showed high levels of aspecific hybridization.

The use of flow cytometry is a new approach to the detection and quantification of DNA sequences in cells. It offers advantages over alternative techniques. Since large numbers of nuclei can be analyzed individually and rapidly, the distribution and relative amount of a DNA sequence in a heterogeneous population can be studied. The instrument used

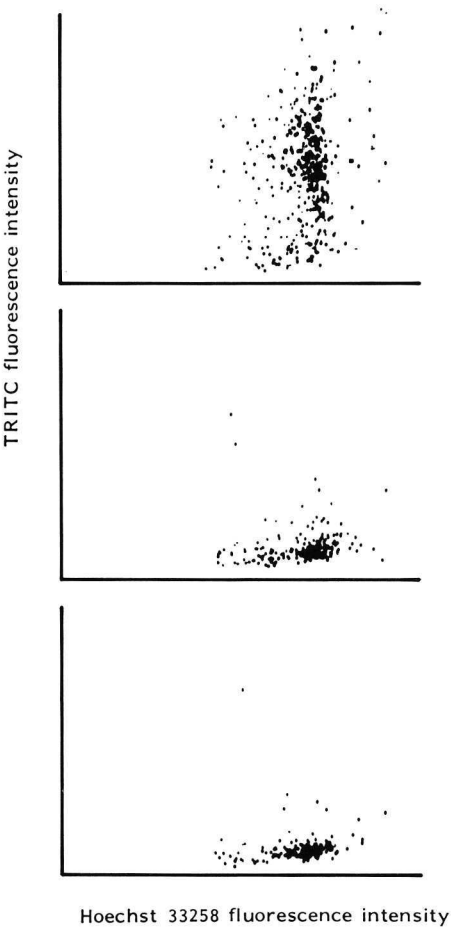


Figure 14.2. Bivariate dot plots showing the TRITC fluorescence intensity and the Hoechst 323258 fluorescence intensity of nuclei hybridized in suspension and measured in a dual beam flow cytometer. Each dot represents the measurement of a single nucleus. Nuclei hybridized with A) mouse satellite DNA probes; B) with an heterologous human DNA probe; or C) in the absence of either probe. All three nuclei suspensions were subjected to immunofluorescent labeling procedures. Machine settings were identical during the measurements in all panels.

in these studies is not particularly sophisticated, and more sensitive machines are available (Dean and Pinkel, 1978). Even so, the median of the fluorescence distribution of nuclei hybridized with a probe complementary to 10% of the total genome is approximately 20 times higher than that of nuclei hybridized with a heterologous probe. This implies that DNA sequences occurring at frequencies as low as 1% should be readily detectable in this system. This level of sensitivity may allow the detection and enumeration, in interphase nuclei, of a given chromosome (given the availability of a mixture of chromosome-specific DNA sequence probes (Davies et al., 1981)), amplified genes, or viral sequences.

The studies presented here may lead to the quantification of specific DNA sequences in individual chromosomes using flow cytometry. Efforts to apply the techniques described in this article to chromosomes in suspension are in progress. Preliminary results show that chromosomes can also be stabilized with DMS (chapter 13). The chromosomes can be denatured with preservation of a normal karyotype as measured in a flow cytometer. The

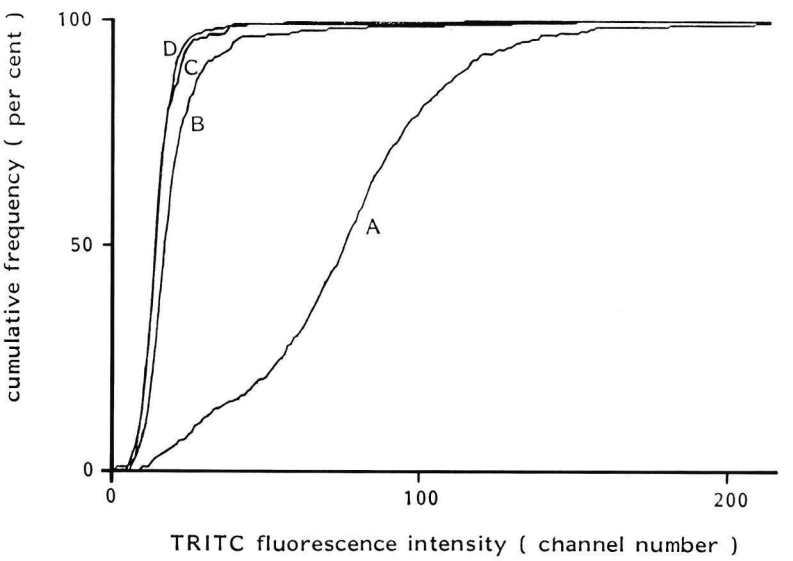


Figure 14.3. Cumulative frequency curves of the TRITC fluorescence intensity of nuclei hybridized in suspension as described in the legend of figure 14.2. The ordinate gives the sum of the number of nuclei with a TRITC fluorescence intensity less than or equal to the channel number indicated on the abscissa. The sum is expressed as a percentage of the total number of nuclei analyzed in each group. A) Mouse thymocyte nuclei hybridized with mouse satellite DNA probe; B) with human DNA probe; C) or without probe. The nuclei in A, B, and C were subjected to antibody labeling procedures. These groups are the same as those displayed in figure 14.2. Mouse nuclei in D were not treated with antibodies.

ability of an antibody, which recognizes bromodeoxyuridine (BrdU) only in single-stranded DNA (Dolbeare et al., 1983; Gratzner, 1982), to bind to BrdU-containing chromosomes indicates that strand separation takes place in these chromosomes (chapter 13). Using sensitive three laser flow cytometers (Van den Engh, manuscr. in prep.), it should be possible to measure the hybridization of DNA probes and, at the same time, identify the chromosomes involved.

MATERIALS AND METHODS

Nuclei isolation and DMS treatment

Nuclei were isolated from the thymocytes of 6-8 wk old BC3 female mice using the isolation procedure described in chapters 7 and 8. For DMS treatment, K_2CO_3 and DMS were added to a suspension containing $5x10^6$ nuclei/ml from a 5x concentrated stock solution mixed immediately before use. Final K_2CO_3 and DMS concentrations were 20 mM and 3 mM, respectively. The pH of the suspension during DMS treatment was 10. After 15 min at 25°C, the pH was adjusted to 8.0 by the addition of 50 µl 100 mM citric acid per ml nuclei. Nuclei were washed once in IB (50mM KCl, 5 mM Hepes, at pH 8.0) containing 10 mM $MgSO_4$ by centrifugation (300g, 10 min, 4°C).

Effect of denaturation conditions on DNA distribution

10^6 nuclei were centrifuged in 1.5 ml Eppendorf tubes (8000g, 2 min) and resuspended in IB containing the additions indicated in table 14.1 at the following concentrations: $MgSO_4$: 10 mM; 2xSSC (1xSSC: 0.15 M NaCl plus 0.015 M sodium citrate); formamide: 50% (v/v); or in 0.07 N NaOH. The nuclei were incubated at the temperatures indicated (where not specified: 1 h, 25°C), centrifuged as above, and resuspended in IB+M (IB containing 10 mM $MgSO_4$). The suspensions were syringed repeatedly through a 23-gauge needle using a 1-ml syringe, filtered through 50 µm nylon mesh, and stained for DNA content using 2 µg/ml Hoechst 33258. Fluorescence intensity distributions were measured in a FACS II (Becton-Dickinson, Sunnyvale, CA) flow cytometer using 100 mW of excitation light in the UV (351+364 nm; Coherent Innova 90 laser (Palo Alto, CA) and a KV 418 (Schott, Mainz) emission filter.

In situ hybridization in suspension

To $2x10^4$ DMS-treated nuclei (130 ng total DNA (MacGregor, 1968) suspended in 20 µl IB in 1.5 ml Eppendorf tubes, the following solutions were added: 28 µl 100% formamide, 5.6 µl 20xSSC, 2 µl of a 10 mg/ml solution of salmon sperm DNA, and 1 µl AAF-modified DNA probe in 10 mM Tris-HCl, 1 mM EDTA, at pH 7.6. Mouse satellite DNA isolated from liver nuclei and total human DNA extracted from placenta were modified with AAF as described (Landegent et al., 1984). The stock solutions of AAF-modified mouse satellite DNA and of AAF-modified human DNA contained 265 ng and 250 ng DNA/µl, respectively, resulting in a probe to complementary sequence ratio of 20. The mixtures of nuclei and probe were denatured by incubation at 70°C for 10 min. Hybridization was carried out at 37°C for 16 h. The nuclei were washed by the addition of 1.0 ml hybridization buffer (50%

formamide/2xSSC/20mM KCl/2 mM HEPES) at 25°C and 100 µl DMS-treated mouse erythrocytes (RBC, see below). After 15 min agitation at 25°C, the nuclei were centrifuged 2 min at 8000g. The pellet was resuspended in 1.5 ml hybridization buffer by repeated syringing through a 23-gauge needle and a 1-ml syringe, centrifuged, and washed in 1.5 ml IB+M.

Immunofluorescent labeling

After hybridization, the nuclei were incubated 60 min at 25°C in 200 µl IB+M containing 2% normal goat serum (NGS, Nordic, Tilburg, The Netherlands) and 1:500 dilution of a rabbit anti-AAF (Baan et al., 1983). The nuclei were suspended in antibody solution by syringing. The nuclei were washed once in IB+M and then incubated 60 min at 25°C in 200 µl IB+M containing 2% normal goat serum and a 1:50 dilution of a TRITC-conjugated goat anti-rabbit IgG (Nordic). The nuclei were washed once and then resuspended in 1.0 ml IB+M by syringing five times as above. They then were filtered through 50 um nylon mesh, stained with 2 µg/ml Hoechst 33258 and measured in a flow cytometer.

DMS-treated erythrocytes

Blood was collected into heparin by cardiac puncture from BC3 female mice. After removal of serum and white blood cell layer, the erythrocytes (RBC) were washed 4 times in sterile physiological salt solution by centrifugation (200g). RBC were suspended in physiological salt at a concentration of 10^8/ml and treated three times with DMS as described table 14.1, with final DMS concentrations of 3 mM, 10 mM and 10 mM, respectively. Additional adjustment of the pH to 9-10 with 100 mM K_2CO_3 was required during the last two treatments. The RBC were washed once in IB and resuspended in IB at a concentration of 10^8/ml.

Slide preparation and photography

After denaturation, hybridization and immunofluorescent labeling of the nuclei, a portion of the nuclei were spun onto microscope slides in a cytocentrifuge (Shandon, Cheshire, England) in the following protocol: 50 µl fetal calf serum, 10g, 20 s; 250 µl nuclei suspension, 300g, 3 min; 100 µl 96% ethanol, 300g, 1 min. Microscopic observation was performed with a DIALUX microscope (Leitz, Wetzlar, FRG) using epi-illumination from an HBO 200 W mercury arc (Osram, Berlin). The filter combinations were SP560+BG38+2 mm LP530 excitation filters, 580 nm dichroic mirror + LP590 emission filter for the red rhodamine fluorescence, and 4 mm UG1 excitation filter and 400 nm dichroic mirror + LP435 emission filter for blue Hoechst fluorescence. Photographs were taken with using Kodak technical panfilm

2415. Exposure times were 15-30 s for Hoechst fluorescence and 2.5-3.0 min for rhodamine fluorescence. The original magnification was x630. The actual diameter of the nuclei is approximately 5 μm.

Dual beam flow cytometry

The flow cytometer used for the measurement of hybridized probe to nuclei was a FACS II modified in Rijswijk for dual beam excitation, in which the fluorescent signals from the two beam spots are spatially and chromatically separated. The nuclei and RBC were first illuminated by 300 mW UV light (multiline 351-364 from Spectra Physics laser model 171, Mountain View, CA) for Hoechst 33258 excitation. Hoechst fluorescence was measured through a KV418 (Schott) filter. Electronic gates were set to select single nuclei and exclude RBC, clumps, and debris from further analysis. The second laser (Coherent model CR 6) produced 100 mW light with a wavelength of 515 nm for TRITC excitation. The TRITC fluorescence of each particle identified as a nucleus by its Hoechst fluorescence was collected through a KV 580 (Schott) filter. Photographs were made of the fluorescence intensity measurements of each nucleus displayed in a two-dimensional oscilloscope.

Chapter 15

NUCLEOTIDE SEQUENCE DETECTION IN INDIVIDUAL CELLS AND NUCLEI USING FILTER HYBRIDIZATION AND NONAUTORADIOGRAPHIC TECHNIQUES

SUMMARY

The presence of mouse satellite DNA sequences is demonstrated in cells and isolated nuclei and chromosomes using filter hybridization. The cells or organelles are applied to nitrocellulose filters. There, the DNA is denatured, and hybridization can be performed. The hybridized probe is visualized using an immuno-enzymatic procedure that employs 2-acetylaminofluorene (AAF)-labeled DNA probes. Individual nuclei and cells can be discerned on the filters. This indicates that the detection sensitivity of this method is at least 0.6 pg target DNA. Individual spots can also be seen on filters to which chromosome suspensions have been applied. The potential use of these results for studying the presence of DNA sequences in heterogeneous cell or chromosome populations is discussed.

INTRODUCTION

Hybridization on nitrocellulose filters has been used for the detection of specific nucleotide base sequences in recombinant bacteria colonies (Grunstein and Hogness, 1975) or in DNA isolated from cells (Botchan et al., 1976) or from fractions of chromosomes separated by sorting (Collard et al., 1982; Lebo et al., 1979). Chromosomes can also be sorted directly onto filters (Bernheim et al., 1983; Collard et al., 1985; Lebo et al., 1984). The DNA is denatured and fixed to the filters by baking. The filters are incubated under hybridization conditions with radioactively labeled sequence probes. The locations of silver grains on the filters after autoradiography indicate the presence of target sequences.

Non-autoradiographic methods are also available for sequence detection on filters (Tchen et al., 1984; Landegent, manuscr. in prep.). In the AAF method (Landegent et al., 1984), the sequence probes are modified with 2-acetylamino-fluorene (AAF). After hybridization to the DNA on filters and stringent washing, the bound probes are made visible with an indirect

immunoenzymatic procedure. The filters are incubated first with an anti-AAF antibody (Baan et al., 1982), followed with an anti-immunoglobulin-alkaline phosphatase (AP) conjugate. When the filters are incubated in the substrate for AP, a dark deposit of enzyme end-products forms where hybridization has occurred.

This chapter demonstrates that nuclei, whole cells, and chromosomes can be applied to and denatured on nitrocellulose filters where hybridization can be performed. The nonautoradiographic AAF method is used to demonstrate the specific hybridization of mouse satellite DNA probes to mouse DNA. The nuclei and chromosomes are isolated from cells using a procedure developed for the preparation of these cell components for flow cytometric analysis. Single nuclei and cells can be detected on filters after hybridization. The consequences of this method for determining the frequency of cells, which contain a specific DNA sequence, in a heterogeneous population is discussed.

MATERIALS AND METHODS

Chromosome, nuclei and cell suspensions

Chromosomes were isolated from mouse fibroblast cell cultures derived from the NIH/3T3 cell line. The cells were maintained in exponential growth in Dulbecco's medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Seralab, Sussex, England). Mitotic cells were collected by shake-off after 10 h incubation with 0.1 μg/ml colcemid (Sigma) treatment. Nuclei and cells were obtained from the thymuses of BC3 female mice at 6-8 wks of age. Chromosomes were isolated as described in chapter 6; nuclei were isolated as described in chapter 8 and 14; cells were suspended in phosphate-buffered saline (PBS). Dilution of chromosomes and nuclei were made in IB+M (50 mM KCL, 5 mM Hepes, 10 mM $MgSO_4$); dilutions of cells were made in PBS.

Control DNA

Mouse satellite DNA and total human DNA were isolated as described (Landegent et al., 1984) and diluted to concentrations of 1000 pg and 100 pg/μl in TE (TE=10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Calf thymus DNA modified with AAF served as the control for the immunoenzymatic labeling reaction (250 pg/μl in TE).

DNA sequence probes

Mouse satellite DNA, isolated from mouse liver nuclei, and total human DNA, extracted from placenta, were chemically treated with N-acetoxy-2-acetyl-amino-fluorene as described (Landegent et al., 1984) to modify

approximately 20% of the quanine residues with AAF. Concentration of stock solutions of probe were 250 pg/μl in TE.

Denaturation and fixation to filters

Nitrocellulose filters were soaked in 2xSSC (1xSSC=0.15 M NaCl, 0.015 M Na-citrate, pH 7.0) and air dried. Nuclei, chromosome or cell suspensions were applied to filters in 1 μl volumes using and Eppendorf pipet. After drying, the DNA was denatured and fixed to filters according to the procedure described by Collard et al. (1985). Filters were placed for 2 min on each of a series of Whatman filters dampened in the following solutions: 1) 0.5 N NaOH, 2) 0.1 N NaOH, 1.5 M NaCl, 3) 200 mM Tris-HCl, 5 mM EDTA, pH 7.5. Filters were then placed in 2xSSC for 5-10 min, air dried, and baked at 80°C for 1 h.

Hybridization

Filters were incubated 0.5 h at 60°C in plastic sacks containing 5x Denhardt's solution (1x Denhardt's= 0.02% Ficoll (MW 400,000, Pharmacia), 0.12% polyvinylpyrolidone (MW 40,000, Sigma PVP 40T), 500 μg/ml single strand salmon sperm DNA (Sigma D1626), 3xSSC, and 0.2% normal goat serum (NGS, Nordic, Tilburg) (approximately 500 μl solution per cm^2 filter). The filters were transferred to new plastic sacks containing 1x Denhardt's, 300 μg/ml salmon sperm DNA, 5% dextran sulfate (MW 500,000 Sigma D6001), 3xSSC, and 0.04% NGS at 60°C. After 1 hr at 60°C, the AAF modified probes were added. The probes were denatured immediately before addition to the filters by incubating them 3 min in boiling water bath and then quenching them on ice. The concentration of probe used was 700 ng/ml hybridization solution. After 16 h at 60°C (with mixing), the filters were washed at 60°C with large volumes of the following solutions: 2xSSC, 3x15 min; 1xSSC, 2x10 min; 0.3xSSC, 2x10 min; and 0.1xSSC, 1x10 min. The filter was rinsed at room temperature in 2xSSC and dried by blotting.

Immunoenzymatic visualization

The filters were incubated in a 1:250 dilution of a monoclonal anti-AAF (Baan et al., 1982) in PBS + 2% NGS for 60 min at room temperature. The filters were washed 3 x 10 min in PBS and incubated in goat-anti-mouse-immunoglobulin conjugated to alkaline phosphatase (Nordic) at a 1:500 dilution in PBS + 2% NGS for 1 h at room temperature. The filters were washed 3 x 10 min in PBS. The filters were then added to a solution containing enzyme substrate: 0.33 mg/ml nitro blue tetrazolium and 0.16 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (both Sigma, and dissolved just before use in dimethylformamide). Incubation proceeded at room temperature approximately 0.5 h or until background developed. The

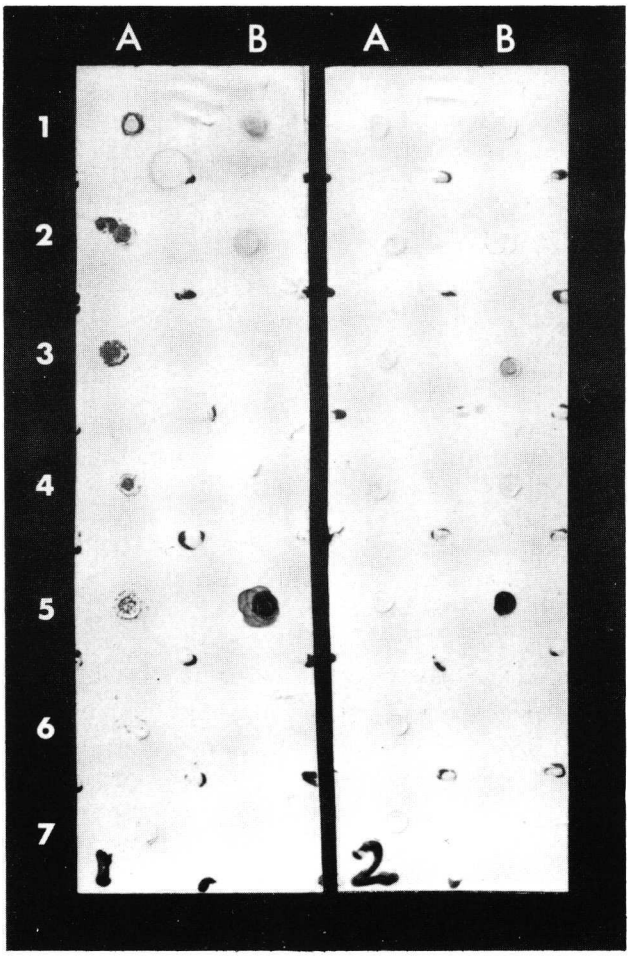


Figure 15.1. Hybridization of mouse satellite DNA probes (left panel) or human DNA probe (right panel) to mouse thymocyte nuclei on nitrocellulose filters. Column (A) in both filters shows 7 areas where the number of nuclei applied ranged from approximately 6200 (top) to 10 (bottom) nuclei (37 ng to 60 pg DNA, respectively) in 1:3 dilution steps. The nuclei were applied in 1 µl volume, denatured as described in Materials + Methods. The specific hybridization signal was visualized with AAF-labeled probe, incubation in anti-AAF, and incubation in a goat-anti-mouse immunoglobulin conjugated to alkaline phosphatase. The dark color represents the formation of enzyme product. The actual size of each application spot is approximately 1 mm. The control spots in column (B) of each filter are: 1) Mouse satellite, DNA 1000 pg and 2) 100 pg; 3) total human DNA, 1000 pg and 4) 100 pg; and 5) calf thymus DNA-AAF, 250 pg.

reaction was stopped in PBS. After the filters were dried, they were photographed. The actual size of pipet tip impression is approximately 1 mm in diameter.

RESULTS

Specific DNA sequences can be detected in nuclei using an immuno-enzymatic filter hybridization method. The hybridization of mouse satellite DNA sequences to mouse thymocyte nuclei was used to demonstrate this method. Nuclei were applied directly to the filters in 1 μl volumes from a dilution series. The DNA was denatured and fixed to the filters as described by Collard et al. (1985). The AAF procedure was used to detect the target sequences (Tchen et al., 1984; Landegent et al., 1984). This procedure employs AAF-modified DNA sequence probes, an anti-AAF antibody, and an anti-immunoglobulin conjugated to alkaline phosphatase. The results are shown in figure 15.1. The positive hybridization signals on the filter incubated in the satellite probe become smaller with increasing dilution of the applied nuclei. No hybridization is observed on the filter incubated in the presence of the heterologous, human DNA probe.

Figure 15.2 demonstrates that mouse satellite sequences can also be detected in whole cells by filter hybridization. Mouse thymocytes were suspended in PBS in a dilution series. Microliter volumes of these suspensions were spotted on filters as described above. As was observed for nuclei, specific hybridization is observed only at the places where the cells are applied if the filters are incubated with the mouse satellite DNA probes. Treatment of the cells with Triton X-100 to solubilize the plasma membrane does not increase the hybridization signal observed.

Single nuclei or cells can be detected on the filters after hybridization with specific DNA sequences using this procedure. Magnified views of several portions of the filters shown in figure 15.1 are displayed in figure 15.3. These photographs show the areas on the filters where approximately 80 (A) or 10 (B) mouse nuclei were applied. Approximately the same number of dark spots of enzyme product, indicating specific hybridization, can be seen on the filters. The actual size of each dark spot is approximately 50 μm. The photograph on the right (C) shows an area of a filter where approximately 80 nuclei were applied. This filter, however, was incubated with human DNA as the sequence probe. Small localized spots of enzyme product are also evident on filters to which low numbers of whole cells were applied (figure 15.2). These spots have been observed on filters where approximately as few as 3 cells or as few as 6 nuclei have been applied. This indicates that the hybridization signal representing individual cells and nuclei is detected.

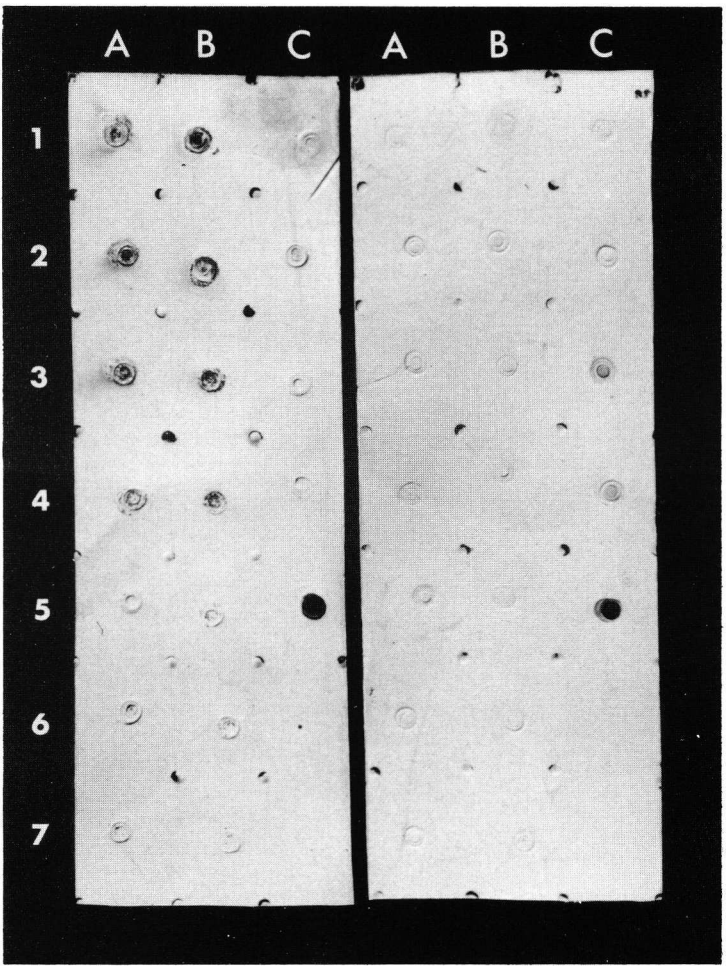


Figure 15.2. Hybridization of whole cells from mouse thymus on nitrocellulose filters with mouse satellite DNA probes (left filter) or human DNA probe (right filter). Column A in both filters shows 7 areas where the number of cells applied range from approximately 3000 (top) to 3 (bottom) (18 ng to 18 pg total DNA, respectively) in 1:3 dilution steps. The nuclei in column B were treated with 0.05% Triton X-100 15 min before spotting. The control spots in column C and the hybridization procedure are as described in the legend to figure 15.1.

Mouse satellite sequences can also be detected in chromosomes using the AAF immunoenzymatic procedure (Figure 15.4). This figure is a magnified view (actual diameter of pipete impression approximately 1 mm) of an area of a filter where 1500-2000 isolated mouse chromosomes were applied. As on the filters of entire nuclei and cells, localized spots of enzyme product are visible on this filter.

DISCUSSION

The results show that nuclei, cells and chromosomes can be applied to nitrocellulose filters where the DNA they contain can be probed for

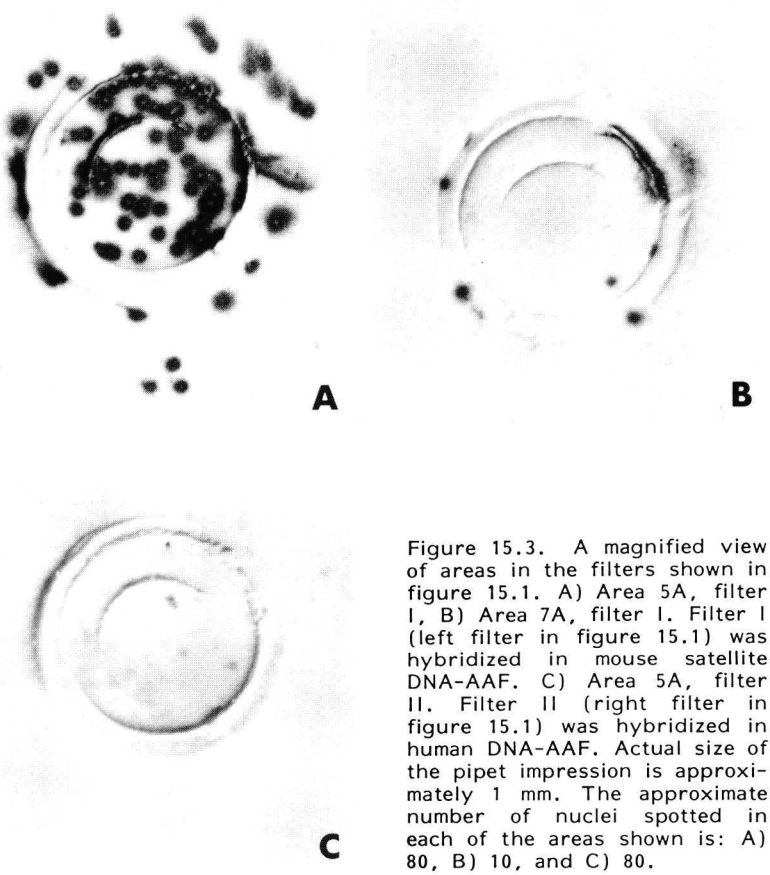


Figure 15.3. A magnified view of areas in the filters shown in figure 15.1. A) Area 5A, filter I, B) Area 7A, filter I. Filter I (left filter in figure 15.1) was hybridized in mouse satellite DNA-AAF. C) Area 5A, filter II. Filter II (right filter in figure 15.1) was hybridized in human DNA-AAF. Actual size of the pipet impression is approximately 1 mm. The approximate number of nuclei spotted in each of the areas shown is: A) 80, B) 10, and C) 80.

specific nucleotide base pair sequences. Single cells and nuclei can be visualized on the filters after hybridization. This was demonstrated using mouse satellite DNA as a sequence probe and an indirect immunoenzymatic hybridization procedure.

The detection of hybridization to a single nucleus or cell implies that the sensitivity of this method is at least 0.6 pg target DNA. Mouse cells are known to contain approximately 6 pg DNA (MacGregor, 1968), Satellite sequences comprise approximately 10% of the total genome (Waring and Britten, 1966). This detection level is approximately an order of magnitude better than that reported for this procedure with isolated DNA (Tchen et al., 1984; Landegent et al., manuscr. in prep). This is presumably a result of the small area in which the DNA is concentrated here (each spot occupies an area 400 times smaller than the application area) and the build-up of enzyme products (50 μm spot for each nucleus).

Autoradiographic methods, however, are capable of a sensitivity approximately 20 times higher than the level observed here (Collard et al., 1985; Bernheim et al., 1983; Lebo et al., 1984). Single genes have been localized with radioactively-labelled probes to chromosomes sorted onto

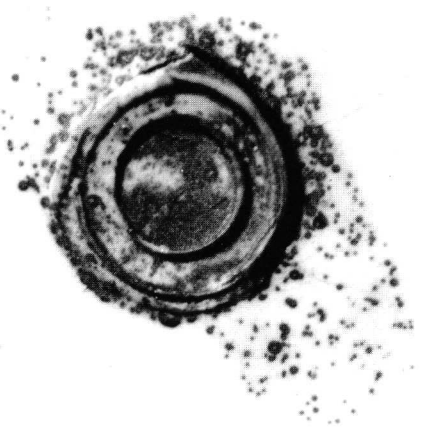


Figure 15.4. A magnified view of a portion of a nitrocellulose filter where approximately 1700 isolated mouse chromosomes were applied. The actual size of the pipet impression is approximately 1 mm. The chromosomes were isolated from mouse fibroblast cultures with the Hepes/$MgSO_4$ method as described in chapter 6. After the chromosomes were spotted onto the filter, the DNA was denatured and hybridized with mouse satellite DNA sequences modified with AAF, as described in Materials + Methods. The target sequences were visualized with anti-AAF and an anti-immunoglobulin conjugated to alkaline phosphatase. The dark color on the filter represents enzyme product formation. The same area on a duplicate filter that was hybridized with human DNA probe-AAF showed no dark spots (not shown).

filters (Collard et al., 1985; Lebo et al., 1984). Indirect immunoenzymatic methods have advantages in rapidity and safety, however. Furthermore, the underlimit for the detection of DNA sequences in individual nuclei has not yet been determined. Probes that are complementary to smaller portions of the total genome will be employed in subsequent experiments.

Small spots of enzyme product are also visible on filters where chromosomes have been applied. Experiments are in progress to sort chromosomes onto the filters to determine if these spots represent the hybridization of mouse satellite DNA to individual chromosomes, clumps of chromosomes or nuclei. If these spots represent individual chromosomes, the detection sensitivity of this nonautoradiographic method is approximately comparable to that of autoradiographic methods.

The localized concentration of the DNA from individual nuclei may be a result of the proteins present in chromatin. These proteins may attach in some way to the filters and prevent the DNA from migrating in the filter. Extracted DNA used as control contains no proteins. This DNA shows a tendency to spread from the spots where it was applied, when the filters are placed on the denaturing solutions. No hybridization signal is detected if nuclei or chromosome suspensions are denatured in an alternative procedure before being applied to the filters (3 min incubation in a boiling water bath). Control mouse satellite DNA, in contrast, shows a high hybridization signal localized to the spot of application, if denatured in this way. This difference may also indicate that the denatured proteins in the suspension of nuclei and chromosomes compete with the DNA for binding to the filters.

The results also show that chromosomes isolated using the Hepes/ $MgSO_4$ method (chapter 6) can be sorted onto filters for subsequent study with DNA sequence probes. This has been reported for chromosomes isolated using other preparation procedures (Collard et al., 1984; Lebo et al., 1984; Bernheim et al., 1983). Chromosomes can be isolated using this protocol from a variety of cell types, including the cells in amniotic cultures, to yield high chromosome resolution in DNA fluorescence distributions measured in a flow cytometer (chapters 7,9).

The ability to visualize individual cells on filters may have several applications in determining the frequency of cells that contain a specific DNA sequence in a heterogeneous population. It may be possible, for example, to determine the frequency of cells in a population that are infected with viral particles or that have incorporated a specific DNA sequence after gene transfer procedures. Using flow cytometers, subpopulations of cells could be sorted, based on cell surface markers or size criteria, to create filter panels. Hybridization could be used to

detect the presence of certain DNA sequences and to determine the frequency of cells that contain these sequences in each subpopulation. This may be an aid to the rapid identification of the chromosomes responsible for cell surface markers expressed in somatic cell hybrids.

Filter hybridization is limited, however, in that it is difficult with this method to quantify the extent or number of copies of specific DNA sequences. A procedure with which specific DNA sequences can be detected in nuclei in suspension was described in chapter 14. With this technique, the hybridization signal of each individual nucleus in a heterogeneous population can be quantified in a flow cytometer.

Chapter 16

GENERAL DISCUSSION

In the history of the study of chromosomes, the microscope has played a dominant role. With a microscope, the morphology--the shape, size, and banding pattern--of chromosomes can be observed. Although flow cytometric measurements will never replace the beautiful pictures of chromosomes obtained with a light microscope or a scanning electron microscope, this technique has the advantage that it provides objective and reproducible criteria for the study of chromosomes. In flow cytometry, the biological properties of chromosomes can be analyzed in a quantitative manner. These properties are translated, via fluorescent labels and electronic circuitry, into electric signals. The electric signals can be quantified accurately and at a high speed. A large number of particles can be inspected in a short time. The flow cytometer is also capable of quantifying more than one optical property for each particle.

Because the flow cytometric measurement is an abstraction of the original chromosomal property, techniques must be developed to ensure that the electric signals contain biologically relevant information. Using the techniques for sample preparation, that are described in this thesis, detailed information can be derived about the molecular organization of chromosomes and nuclei. A large range of properties can be quantified. One can determine the total DNA content, the base composition, and the protein composition of chromosomes. The conformational state of the DNA and of the nucleoprotein complex can also be studied by flow cytometry. A newly developed technique, which has perhaps the greatest potential, makes it possible to quantify specific nucleotide sequences in individual chromosomes and nuclei. These procedures have also been perfected so that they can now be performed on a variety of cell types. Previously, chromosomes could only be obtained for flow cytometric analysis from rapidly proliferating cell lines. Procedures are now available to produce good chromosome suspensions from a few thousand cells or from cell types that have a low proliferation rate. It is possible to start applying these techniques to clinical investigations. It is in this area that the high speed and the capacity to rapidly and objectively analyze large numbers of samples may be of enormous advantage. The data on amniotic cell cultures that are presented in this thesis show that such applications are possible.

The potential of flow cytometry for extracting useful information from a cell or chromosome preparation is a combined function of sample preparation, the staining procedures and the flow cytometric equipment. This thesis presents an evaluation of these three factors and methods for their improvement.

Improvements in the chromosome isolation procedure have been demonstrated here. High resolution can be obtained in the fluorescence distributions of chromosomes from a variety of cell types. When the isolation procedure is applied to human cells, a flow karyotype is obtained that is reproducible and permits consistent discrimination of almost all the human chromosome types. Differences between the homologues of some chromosomes are also detectable. The chromosome suspensions can also be used as the starting point for other biochemical studies. The isolated chromosomes are stable. High molecular weight DNA can be extracted from the chromosomes. The chromosomes are morphologically intact. They can be stained in a variety of ways. In combination with the high flow karyotype resolution, these features provide a good basis for the purification of chromosomes for further biochemical studies.

Staining procedures control the properties of chromatin that can be studied quantitatively in flow cytometry. The studies in this thesis have broadened the range of fluorescent assays that can be applied to chromosomes, as well as nuclei, in suspension. The interaction and binding of base-pair specific DNA ligands can be quantified. The accessibility and

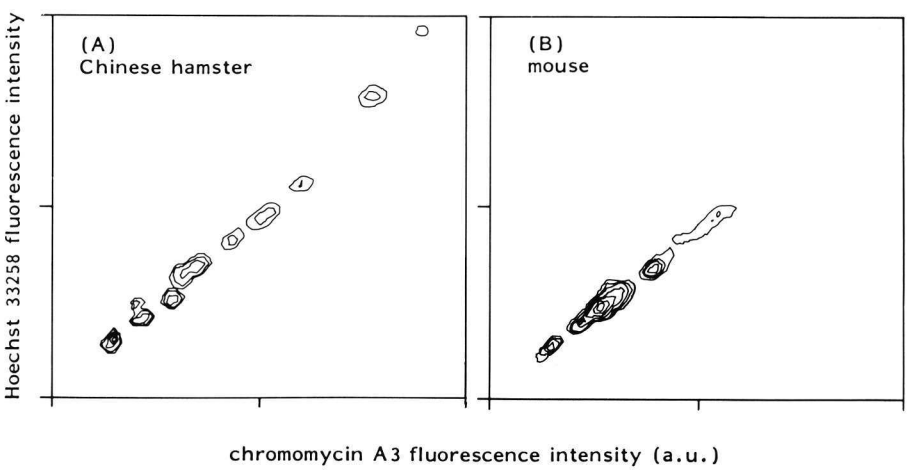


Figure 16.1. Bivariate distribution of Hoechst 33258 vs. chromomycin A3 fluorescence of chromosomes isolated from (A) Chinese hamster culture cells or (B) mouse culture cells.

binding of antibodies specific for structural proteins or nucleotides can be studied. Finally, in situ hybridization techniques can be combined with fluorescent labeling procedures to assay specific nucleotide sequences in individual nuclei. It is probable that many other labeling procedures that are used for staining chromosomes on microscope slides can be adapted to chromosomes in suspension and can be added to the list of properties that can be quantified with flow cytometry. Several of these possibilities are discussed later in this chapter.

The ability to detect staining differences in chromosomes or nuclei, even if they are optimally prepared and fluorescently labeled, is often limited by the performance of the flow cytometer and the mode of data acquisition. The highest resolution in the studies presented here was attained on the dual beam flow cytometer in Livermore. This system incorporates several features that contribute to high sensitivity and resolution. Slow and stable sample flow through a quartz cuvette allows accurate measurement of passing particles. The lasers are focused into a small light spot for high illumination intensity. The collection optics were carefully designed and combine spatial and spectral separation of the fluorescent signals. The electronic circuitry combines, among other things, the ability to integrate the area under each signal pulse. These design features make it possible to accurately measure the fluorescence properties of cells or chromosomes.

The techniques are now available that make it possible to express a variety of biological properties of individual chromosomes and nuclei in a quantitative manner. Some of the ways in which the capabilities of flow cytometry for accurate, sensitive, rapid and multiparameter measurement can be used to advantage are demonstrated in this thesis. It is possible to detect small differences in the DNA content of different chromosomes. Differences in anti-histone binding to chromosomes of different species, which are not visible under the microscope, can be detected and quantified with a flow cytometer. When two parameters are measured for each chromosome, an accurate comparison of the antibody binding to chromosomes of the same DNA content can be made.

The advantage of multiparameter analysis is clear from the study of human chromosomes. Only some of the human chromosomes are resolved with a single DNA fluorochrome. When the chromosomes are stained with two dyes, almost all the human chromosomes are resolved. These dyes provide complementary information on the base composition of the chromosomes. This does not necessarily hold for other species. This type of analysis provides little basis for the discrimination of mouse chromosomes (figure 16.1B). The chromosomes of the mouse exhibit little heterogeneity in

either DNA content or base composition. New biochemical assays must be developed for the study of the chromosomes of this species to supplement the present battery of stains. If in situ hybridization techniques can be performed on chromosomes in suspension, the presence of specific DNA sequences may provide a basis for chromosome recognition.

The ability to measure more than one parameter per cell also makes it possible to recognize specific events in the midst of irrelevant signals. The analysis of hybridized DNA sequence probes to nuclei (chapter 14) demonstrates this. On the basis of DNA staining, single nuclei can be discriminated from erythrocytes, debris, cell fragments, and aggregates. This is possible despite the fact that the nuclei are mixed with these other particles at a ratio of less than one to a thousand. The nuclei are selected before analysis of the fluorescent signals indicating hybridized probe. Whether the erythrocytes or debris particles aspecifically bind the DNA sequence probe or the fluorescent antibodies or not is irrelevant.

The high rate of analysis increases the chance that cells or chromosomes that are present at low frequencies in a population can be detected within a reasonable period of time. The example in the previous paragraph illustrates one situation where this feature of flow cytometry is useful. It is also an advantage over conventional techniques for the detection of infrequently occurring chromosomal defects (chapter 7).

There are many areas in cell biology where the results of the studies described here could be applied. Some of these areas are summarized in the following paragraphs.

Flow karyotyping holds promise for the detection of chromosomal abnormalities. As the techniques to measure chromosomal properties improve, it is likely that a more detailed picture will emerge of the association of specific chromosomal abnormalities with certain tumors or disorders than that already available from conventional banding analysis. A protocol has been described here for staining chromosomes to produce conventional banding patterns after they have been isolated and studied in a flow cytometer (chapter 11). This protocol should help in making the link between the chromosomal defects detected flow cytometrically and those detected conventionally.

Defective chromosomes can be purified by sorting for further analysis, if they can be sufficiently resolved in a flow karyotype. For example, they can be sorted onto nitrocellulose filters where their nucleotide sequence can be probed. Alternatively, DNA from abnormal chromosomes can be cloned to produce chromosome specific gene libraries. The DNA fragments

from the defective region in the chromosomes can be used to study the molecular nature of the disorder and its biological consequences.

At present, the limiting factor in this application is the preparation of chromosome suspensions of sufficient quality from tumor cells. Methods for the optimal dissociation of tumor tissue, and perhaps the short-term culture of tumor and leukemic cells, are needed for the isolation of chromosomes from these tissues. The enrichment of mitotic cells in rat bone marrow suspensions is promising in this regard. This technique eliminates one of the hurdles that must be cleared before the analysis of the chromosomes from this tissue can be accomplished.

Univariate fluorescence distributions of the chromosomes from several in vitro human leukemic cell lines have been presented by Wirchubsky et al. (1984). The results in this thesis show that much greater resolution can be expected if two parameters are analyzed for each chromosome. This resolution should make it more likely that deviations in the flow karyotype can be detected. Changes in peak position or peak area contain quantitative information about the normal and abnormal chromosomes. Reciprocal translocations may be reflected in changes in the HO/CA3 fluorescence ratio. The appearance of small new peaks in a flow karyotype may indicate the presence of abnormal chromosomes within a population of normal chromosomes.

If suitable techniques can be developed for preparing chromosomes from tumor tissue, it may be possible to compare the flow karyotype of normal and tumor cells from the same patient. It may also be possible to determine the number of residual tumor cells remaining after cancer therapy, by comparing flow karyotypes before and after treatment.

Another application for flow karyotyping is the detection of chromosomal abnormalities induced in the cells of persons exposed to mutagenic agents, such as radiation. The chromosomal defects caused by these agents tend to be random, and they occur infrequently in the cell population. The main consequence of these changes is the appearance of a background continuum underlying the peaks and filling the valleys in the flow karyotype. In chapter 7, the flow karyotypes from peripheral blood lymphocytes were presented. This cell type is perhaps the most suited for population screening studies. The cells are easy to obtain and can be grown for several days in the laboratory. The low amount of debris between the peaks in the karyotypes obtained from these cells forms a sound foundation for the detection of heterogeneous chromosomal abnormalities.

Slit scan flow cytometry provides an alternative means for the detection of infrequently occuring chromosomal defects (Lucas et al., 1983;

Gray et al., 1984). With this tool, the frequency of dicentric and acentric chromosomes in a chromosome suspension can be determined rapidly.

The third main application of flow karyotyping is in the detection of chromosomal defects in fetal cells. Amniotic cell cultures have been historically classified as a "difficult" starting material for the preparation of chromosome suspensions. With previous chromosome isolation procedures, more than a million cells were required for optimal flow karyotype resolution. The results in chapter 9 show that the procedures can be scaled down radically. It is now possible to prepare and analyze the chromosome suspensions obtained from the low number of mitotic cells in these cultures. With these techniques, trisomy of chromosome 21 has been detected flow cytometrically (figure 16.2) and has been confirmed cytogenetically. Fetal sex can also be determined rapidly. Amniotic cells are now being analyzed on a routine scale in the Livermore laboratory. With this study, the detection capabilities of flow karyotyping can be compared to those of conventional banding analysis.

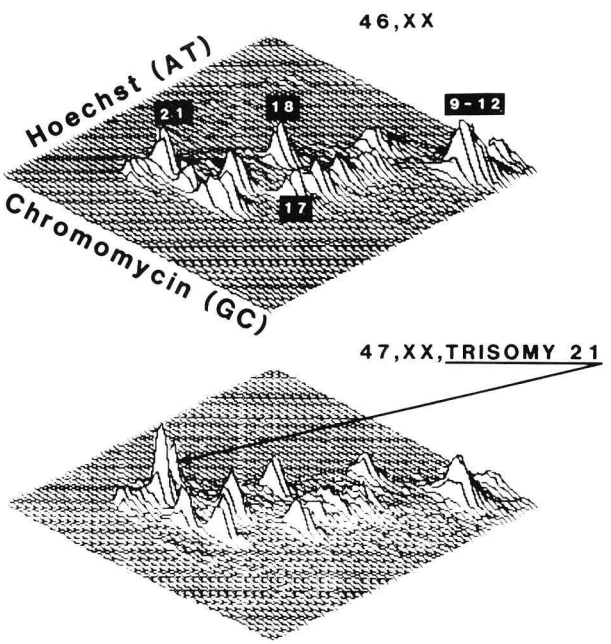


Figure 16.2. Bivariate distribution of Hoechst 33258 vs. chromomycin A3 fluorescence of chromosomes isolated from (A) a normal human amniotic cell culture, and (B) an amniotic cell culture from a fetus with Down's syndrome (three, instead of 2 copies of chromosome 21) (figure compliments of J. Gray, Livermore).

New techniques are being developed that may eventually replace amniocentesis for the detection of chromosomal abnormalities in fetal cells. With these techniques, a small number of fetal cells, sufficient for conventional analyses, are derived from chorionic villi (Kolata, 1983). Recently, the possibility to measure flow karyotypes from these fetal samples has also been made a reality (Yu and Gray, personal communication).

The utility of immunofluorescent reagents for the study of the structural and molecular organization of chromosomes has been demonstrated here. With an anti-histone antibody, the structural proteins in the chromosomes of several species can be assayed. With an antibody specific for bromodeoxyuridine, this nucleotide can be quantified in individual chromosomes. In addition, these tools can be used to study the conformation of the DNA helix and its complex with structural proteins. These findings make the abundance of specific monoclonal antibody reagents available for the study of the composition and conformation of individual chromosomes.

The conformation structure of chromosomes could be probed, for example, with antibodies specific for other structural proteins. The changes in the chromosome structure caused by the insertion of intercalating dyes could be further assessed using these antibodies.

Other antibodies are available that specifically recognize some of the lesions produced in DNA after the exposure of cells to chemical mutagens or irradiation (for example, anti-pyrimidine dimers, anti-AAF). These molecular probes could be used in combination with flow karyotyping to quantify the number of lesions in each chromosome.

Antibodies specific for single-stranded DNA may be useful in studying the distribution of regions in the genome that rapidly reassociate after denaturation (satellite regions). In addition, conformational differences are known to exist between areas of chromatin near active genes and those near quiescent genes. With suitable fluorescent tools, it may be possible to detect and quantify these areas in the chromosomes from different cell types.

Differences in the time of replication among different chromosomes have been detected using differential BrdU uptake (Cremer and Gray, 1983). With careful administration of BrdU to synchronized cell populations, it could be possible to detect sister chromatid exchange in chromosomes using antiBrdU labeling, while identifying the chromosomes involved using DNA stains.

Specific sequences of nucleotide bases can be quantified in the DNA of nuclei. Two methods for sequence detection are presented in this

thesis. In one, nuclei or cells are fixed to filters where hybridization of specific DNA probes can be performed. In the second, the nuclei are labeled in suspension using fluorescent in situ hybridization. Both techniques could be used to determine the frequency of cells containing specific DNA sequences within a cell population. In situ hybridization in suspension has the obvious advantage, however, that the bound probe can be quantified in a flow cytometer.

The quantification of hybridized sequences to nuclei using flow cytometry could be applied in a variety of studies. Numerical chromosome disorders, or aneuploidy, could be detected in cells without stimulating them to divide. For example, a collection of DNA sequence probes for chromosome 21 could be hybridized to interphase nuclei from amniotic cell cultures. In this way, it may be possible to diagnose Down's syndrome without ever seeing a chromosome. Important for this application is the fact that the techniques have been developed for the application of in situ hybridization to as few as 10^4 cells.

If the methods prove sufficiently sensitive, it may be possible to detect and quantify the insertion of viral sequences in the genome of infected cells. The frequency of cells containing gene sequences inserted through gene transfer procedures might also be determined using these techniques.

If three laser flow cytometers are available, nuclei could be hybridized to two different sequence probes carrying different fluorescent labels. The target sequences of both probes, in addition to the total DNA content, could be quantified for each nucleus. If the spectral characteristics of the fluorescent labels overlap, it may be possible to study the proximity of the probes using resonance energy transfer.

Filter hybridization has a potential application in detecting DNA sequences in individual cells. Flow cytometers can be used to accurately sort chromosomes, cells or nuclei to specific sites on filters, to create a filter panel. Using suitable sequence probes, the frequency of cells containing specific DNA sequences, for example viral genes, could be determined. It is also possible to separate and sort specific cell populations onto filters. These filters could be used to study the distribution of specific sequences among cell populations. For example, amplified gene sequences have been associated with tumor cells. In situ hybridization together with flow cytometry may be a means to quantify the degree of gene amplification in different populations of cells.

The expression of the genome, in addition to its organization, is of importance in understanding cell differentiation and cell function. The expression of certain genes can be used to detect tumor cells. Flow

cytometry and in situ hybridization may also have a role in the study of gene expression. It is already possible to quantify specific mRNA's in cells on slides using fluorescent labels (Singer and Ward, 1982), Venezky et al., 1981; Lynn et al., 1983; Brahic et al., 1984). It seems feasible to sort subpopulations of cells onto filters and to assay them for specific RNA sequences. Alternatively, if suitable cell fixation techniques can be developed, it may be possible to perform in situ hybridization in whole cells to quantify RNA sequences using flow cytometry.

In this thesis, the foundations have been laid for performing in situ hybridization on chromosomes in suspension. Chromosomes can be stained using immunofluorescent labels and DNA stains. Of greatest importance is that, using special fixation techniques, the DNA of chromosomes in suspension can be denatured to form single strands without losing the integrity of the chromosome itself. The last obstacle appears to be methods to wash chromosomes repeatedly and stringently, without their clumping. If the staining procedures are successful, rapid gene assignment to chromosomes could be made in a flow cytometer. If fluorescent in situ hybridization techniques can be combined with bivariate flow karyotyping, the chromosomes to which the gene probes bind could be identified. Using a collection of chromosome specific probes, portions of chromosomes could be identified even if they were translocated to new positions. It may also be possible to quantify in this way the amount of DNA involved in the translocation. Alternatively, gene probes could be hybridized to filter panels containing each of the human chromosome types sorted from normal and, for example, leukemic cells. This could also be a rapid means for studying chromosomal translocation. Techniques for in situ hybridization on chromosomes could be combined with slit scan flow cytometry. With this tool, it may even be possible to locate gene sequences to specific positions in the chromosomes.

In conclusion, the studies described in this thesis cover many levels of the organization of chromatin. This series of experiments has progressed from the quantification of the DNA content, the base composition, the conformation of the DNA, the structural proteins, specific nucleotides, and finally to the quantification of specific nucleotide sequences. In all these studies, the flow cytometer has proven to be an ideal tool for the objective, quantitative, and rapid measurement of chromatin properties. Future studies may produce additional biochemical properties that can be analyzed. Application of these techniques to clinical investigations may reveal whether they can be used as diagnostic tools.

SAMENVATTING

Dit proefschrift behandelt het gebruik van doorstroom-cytometrie (flow cytometry) voor het bestuderen van de struktuur en moleculaire organisatie van kernen en chromosomen. Er worden verschillende manieren beschreven waarop chromatine met een fluorescerende stof gemerkt kan worden, waardoor een kwantitatieve meting met een doorstroom-cytometer mogelijk wordt.

In het eerste deel wordt achtergrondinformatie gegeven over de doorstroom-cytometrie en de analyse van chromosomen. De introductie van de principes van de doorstroom-cytometrie vindt hier plaats. Het nut van de doorstroom-cytometrie voor de analyse van de samenstelling van chromosomen wordt samengevat. Factoren van belang voor de resolutie van de metingen en de kwaliteit van kleuringen worden aangegeven.

In het tweede deel worden experimenten beschreven waarin de doorstroom- cytometrie wordt gebruikt voor het onderscheiden van chromosomen, waarbij flowkaryotypen getoond worden van een reeks menselijke cellijnen en cellen van de Chinese hamster. De totale DNA-inhoud en de nucleotide samenstelling zijn gebruikt om de verschillende chromosomen te kunnen indentificeren. Enkel- en dubbel-laser doorstroom-cytometers werden gebruikt om histogrammen en bivariate distributies samen te stellen. Deze hoofdstukken demonstreren dat de kwaliteit van de metingen afhankelijk is van de wijze waarop de chromosomen geïsoleerd worden. Een nieuwe procedure is beschreven voor de preparatie van een stabiele chromosoom suspensies, waarvan een nauwkeurige flow-karyotype gemaakt kan worden. Dit deel beschrijft de kinetiek van de DNA-binding van een aantal fluorescerende kleurstoffen.

Het derde deel behandelt de struktuur van individuele chromosomen. De grootte van DNA-fragmenten die uit geïsoleerde chromosomen geëxtraheerd werden wordt beschreven. Aangetoond wordt dat op geïsoleerde chromosomen banderingspatronen zichtbaar gemaakt kunnen worden. Een struktureel eiwit, histoon 2B, wordt aangetoond door middel van een monoclonaal antilichaam. Deze experimenten geven inzicht in de struktuur van het eiwit/DNA-complex van chromosomen.

In het vierde deel zijn de chromosomen en kernen beschreven op het niveau van de nucleotide volgorde in het DNA-molekuul. Technieken worden beschreven voor het stabiliseren van chromosomen en kernen in suspensie. Deze stabilisatie maakt het mogelijk chromosomen en kernen te onderwerpen aan omstandigheden waarin het DNA gedenatureerd en "high stringency" DNA-DNA-hybridisaties plaatsvinden. De binding van een antilichaam,

gericht tegen bromodeoxyuridine in DNA-ketens, dient om de mate van ketenseparatie te kwantificeren. In situ hybridisatie technieken worden toegepast op de kernen van interfasecellen in suspensie. Na het merken van muize-satelliet-DNA door middel van immunofluorescentie wordt de binding van dit DNA in muizekernen aangetoond in een doorstroom-cytometer. Een alternatieve manier voor het aantonen van specifieke nucleotidereeksen in kernen en chromosomen wordt eveneens gepresenteerd. Chromosomen en kernen worden aan nitrocellulosefilters gehecht, waarna DNA-hybridisatie plaats vindt. Hybridisatie kan met een immuno-enzymatische methode worden aangetoond. De experimenten laten zien dat het mogelijk is op deze wijze een enkele celkern of zelfs een chromosoom zichtbaar te maken.

Bij de bestudering van de verschillende niveaus van de organisatie van het genoom is doorstroom-cytometrie een ideaal hulpmiddel. De mogelijkheden doorstroom-cytometrie toe te passen bij de studie en diagnose van genetische abnormaliteiten en neoplastische afwijkingen van de mens worden vergroot door de technieken die in dit proefschrift beschreven zijn. In dit opzicht zijn de resultaten verkregen met chromosomen van foetale cellen uit amniotische celkweken en van kweken van perifere bloedlymfocyten, veelbelovend. Een methode waarmee de mitotische cellen in rattebeenmerg geconcentreerd kunnen worden kan ook van nut zijn bij de toepassing van flow-karyotypering van bloed- en beenmergcellen. De technieken ontwikkelt voor de in situ hybridisatie in suspensie kunnen in combinatie met de doorstroom-cytometrie, nieuwe onderzoekswegen openstellen. Detectie en kwantificering van numerieke chromosoomafwijkingen, gen-amplificatie of virale DNA in menselijke cellen worden mogelijk. De hybridisatietechnieken voor cellen in suspensie en op filters kunnen van toepassing zijn bij het bepalen van de incidentie van cellen met een bepaalde DNA-volgorde in een heterogene populatie. Het slot behandelt de mogelijkheden en beperkingen van de doorstroom-cytometrie en celsortering voor deze onderzoeksgebieden.

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ABBREVIATIONS

AAF	2-acetylamino-fluorene
ADC	analog to digital converter
alpha-MEM	alpha-minimum essential medium
anti-H2B	antibody specific for histone 2B molecules
anti-IgM	antibody specific for immunoglobulin M molecules
anti-IgG	antibody specific for immunoglobulin G molecules
AP	alkaline phosphatase
A-T	adenine-thymidine
BN	brown Norway rat
BrdU	bromodeoxyuridine
CA3	chromomycin A3, a DNA fluorochrome
CV	coefficient of variation
DAPI	4'-6-diamidino-2-phenylindole, a DNA fluorochrome
DBC	dual beam flow cytometer
DIPI	4'-6-bis(2'-imidazolinyl-4H,5H)-2-phenylindole, a DNA dye
DMS	dimethylsuberimidate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EB	ethidium bromide, a DNA fluorochrome
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLS	forward light scatter
G-C	guanine-cytosine
G_1	phase in the cell cycle when cells contain 2N DNA
G_2	phase in the cell cycle when cells contain 4N DNA
HEP	a transformed human fibroblast cell line
HH	Hank's balanced salt solution buffered with Hepes
HO	Hoechst 33258, a DNA fluorochrome
IB	50 mM KCl + 5 mM Hepes
IB+M	IB plus 10 mM $MgSO_4$
LLL-811	a normal human fibroblast cell line
LOVO	an in vitro human colon carcinoma cell line
M	mitotis
MBL	a normal human fibroblast cell line
MPHA	multi-channel pulse height analyzer
Na-citrate	sodium citrate

NBCH	a Chinese hamster fibroblast cell line
NGS	normal goat serum
NIH/3T3	a transformed mouse fibroblast cell line
PBS	phosphate buffered saline
PHA	phytohemagglutinin
PI	propidium iodide, a DNA fluorochrome
PM	photomultiplier
PSH	peak sense and hold circuitry
RBC	erythrocytes
RNA	ribonucleic acid
RNase	ribonuclease
RS	rat rhabdomyosarcoma in vitro culture cells
S	phase in the cell when DNA is being synthesized
SSC	standard saline citrate: 0.15 M NaCl + 0.015 M Na-citrate
TRITC	tetramethylrhodamine isothiocyanate
UV	wavelengths of light in the ultraviolet
WCHE	a Chinese hamster fibroblast line
3-FC	three laser flow cytometer

CURRICULUM VITAE

Barbara Trask was born in Columbus, Ohio, USA, on July 1, 1953. She graduated summa cum laude from Holland High School, Holland, Michigan, in May 1971. From September 1971 to May 1977, she studied at Purdue University, West Lafayette, Indiana. In 1975, she received a Bachelor's degree cum laude in biology with a major in wildlife biology. In 1977, she received a Master's degree in biology after completing a thesis on the behavioral effects of a sexual pheromone in hamsters. In 1977, she moved to the Netherlands. Under the supervision of G.M. Lokhorst at the Rijksherbarium in Leiden, she studied the taxonomy of two green algae species found on the Dutch coast. Under the direction of Prof. Dr. D.W. van Bekkum at the Radiobiological Institute TNO, she studied the antigen expression of the hemopoietic stem cell using flow cytometry. She received her doctoraal degree in 1979. During 1980 at the Radiobiological Institute, she investigated the capabilities of flow cytometry for the discrimination of algae species. This thesis represents investigations carried out from November 1982 to April 1985 at the Los Alamos National Laboratory, Los Alamos, New Mexico, at the Lawrence Livermore National Laboratory, Livermore, California, at the Radiobiological Institute TNO, and at the Sylvius Laboratory, Leiden. In April 1985, she joined the staff of the Lawrence Livermore National Laboratory.