Chapter II

Materials, methods and technical developments

In this thesis each of the following chapters is preceded by a description of materials and methods. This chapter describes in detail how the most important methodologies were developed and used.

2.1 Chromosome studies by flow karyotyping

Flow karyotyping does not differ from conventional karyotyping with regard to the fact that metaphase chromosomes are required. The amount of chromosomes, however, that is needed to obtain a high resolution flow karyogram is much higher. While conventional cytogenetics require approximately 10 to 30 metaphases to determine a karyotype, a flow karyogram is composed of at least 20,000 chromosomes which resembles an equivalent of approximately 450 mitotic cells.

2.1.1 Cell cycle and proliferation

To obtain and study chromosomes cells have to be actively in cell cycle. Going through the cell cycle step by step, the following stages can be discriminated (Figure 2.1). Shortly after cell division cells are in a G1/G0 state. In this stage of the cell cycle, the cellular machinery is set to produce RNA and proteins. No DNA synthesis occurs. Though the difference between G1 and G0 is morphologically not clearly recognizable, it is generally accepted that cells can only traverse through the cell cycle from G1. In both G1 and G0 the amount of DNA is constant in time and designated as 2n (where n is the haploid amount of DNA). At the end of G1, cells enter the synthesis- or S-phase in which RNA and protein production is reduced to a minimum and DNA synthesis takes place. During the period of S-phase the amount of DNA in the cells is increasing gradually. The S-phase ends when the amount of DNA in the cell has doubled as compared to G1. This situation is called G2. In this stage cells contain 4n DNA and they are ready to divide the DNA into two 2n over the two daughter cells.

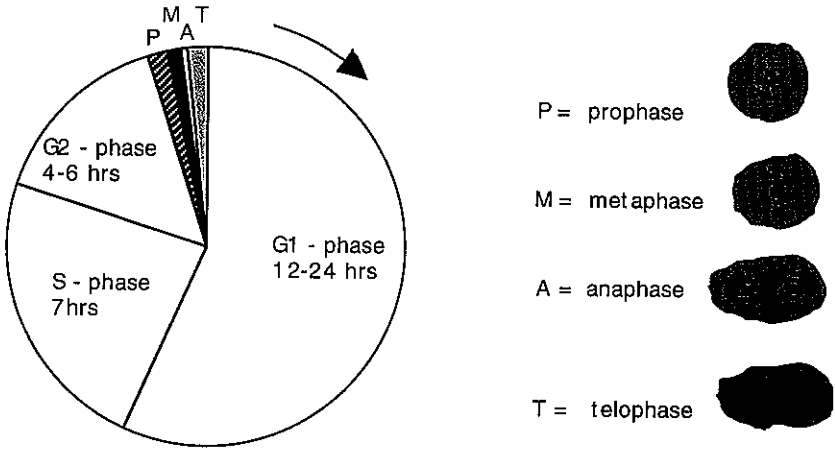

Figure 2.1
The various stages of the cell cycle. Four subsequent stages of the mitotic process are shown. Metaphase is the stage in which chromosomes can be studied.

The cell division starts with disappearance of the nuclear membrane and the condensation of the nuclear DNA into separate chromosomes (referred to as prophase). Microtubuli are being assembled that will finally align the chromosomes at the equatorial plane. This particular stage is called metaphase. The chromosomes consist of two chromatids which are separated and then move towards the opposite poles of the cell (referred to as anaphase). Over the years several models have been proposed that describe the way in which this mechanism takes place (for review see Rieder and Salmon 1994). The two chromatid clusters decondense again while a new nuclear membrane is formed around the two clusters. With the formation of a cell membrane between the two new nuclei the cell cycle is completed. Two daughter cells have been formed, ready to continue in G1 or to leave the cell cycle (temporarily) to enter G0. The duration of the cell cycle varies very considerably from cell type to cell type. Only in a relatively short period of the cell cycle, cells are in metaphase and chromosomes can be studied or isolated. In an average cell cycle, G1 takes 12-24 hours, S-phase 7 hours, G2 4-6 hours. Mitosis takes 1 hour, from which the metaphase is only a part.

To study chromosomes, two requirements must be fulfilled. The first one is to stimulate cells to enter the cell cycle. The second is to accumulate the number of cells in metaphase and extend their period of staying in this state.

2.1.2 Mitotic index

In populations of cells or cell lines that are characterized by a high proliferative index, a high number of cells will continuously pass the mitotic phase in cell cycle. This will result in a high mitotic index (= number of metaphases per 100 nuclei). Leukemic cells in freshly obtained bone marrow samples that were used for flow karyotyping, however, needed to be specifically stimulated to obtain cells in mitosis. Leukemic cells respond to a number of growth factors by proliferation (Griffin et al. 1986, Delwel et al. 1987, Vellenga et al. 1987).

Optimal stimulation of proliferation of leukemic cells depends on subtle differences varying from sample to sample. Since most of the studied cases in this thesis represent newly obtained (diagnostic) material, detailed information about the optimal dosing of the various growth factors was unknown. Therefore it was attempted to find a protocol to which the majority of the leukemic cells would respond by proliferation. For this purpose the leukemias from the myeloid lineage were stimulated by the addition of granulocyte macrophage-colony stimulating factor (GM-CSF) and/or interleukin-3 (IL-3). Recombinant GM-CSF (Behring Werke Ger.) and IL-3 (Biogen, Willemstad, Curacao, Netherlands Antilles) or conditioned media derived from the GCT cell line (DiPersio et al. 1978, DiPersio et al. 1980) were used. GCT cells express IL-1 alpha, IL-1 beta, IL-6, macrophage colony-stimulating factor (M-CSF or CSF-1), GM-CSF and transforming growth factor beta (TGF-beta) as well as both A and B chains of platelet-derived growth factor (PDGF) (Liesveld et al. 1993). It was attempted to stimulate acute lymphocytic leukemia cells with phorbol ester (TPA) or interferon-gamma. Unstimulated and phytohemagglutinin (PHA) stimulated cultures were performed in both myelocytic and lymphocytic leukemias. Furthermore, in a number of cases cell samples were cultured in the presence of a conditioned medium derived from the gibbon T-cell line MLA-144 that produces abundant amounts of IL-2 (Rabin et al. 1981). Cells were grown in alpha modification of minimal essential medium (alpha MEM; Flow Laboratories Irvin, Scotland, UK) at a concentration of 10^6 cells per ml. The medium was supplemented with 2mM L-Glutamine, Penicillin, Streptomycin and 10% fetal calf serum (FCS).

The main objective of the study was to obtain chromosomes in suspension. From the cell suspensions a limited number of additional assays could be performed; i.e. determination of cell recovery after culture and the mitotic index. On the basis of cell counting by eosin exclusion, 10^6 cells per ml were set up in culture flasks (Falcon). The number of living cells was counted after 3 days of culture using eosin exclusion. A cell sample was examined by microscope using a Bürker type hemocytometer. Cells that had not taken up the dye were classified as being alive. The number of living cells was compared to the number of cells that was plated at day zero. The

results, expressed in percentage recovery of living cells after 3 days, are displayed in Table 2.1.

Table 2.1 Recovery of living cells after 3 days of in vitro culture

	AML			ALL			CML		
	n	rec. (%)	stdev	n	rec. (%)	stdev	n	rec. (%)	stdev
GM-CSF	10	**51.0**	30.2						
IL-3+ GM-CSF	39	**79.8**	93.2	5	**72.5**	57.5	14	**59.7**	31.0
GCT-CM (BM)	14	**85.1**	63.6	2	**39.9**	28.1	14	**78.1**	49.1
GCT-CM (PBL)	7	**99.5**	117						
MLA-CM	9	**111.3**	99.6	2	**62.8**	9.5	5	**51.7**	29.3
MLA+GCT	10	**84.8**	84.0				4	**126.8**	118.8
IFN				1	**74.8**	-			
TPA				2	**103.6**	20.2			
IFN +TPA				2	**228.8**	196.9			
PHA	58	**56.5**	47.0	17	**66.8**	30.6	31	**45.6**	31.1
blank	11	**63.6**	43.4	9	**59.4**	41.3	6	**67.8**	42.6

n = number of cases
rec. = recovery
stdev = standard deviation
AML = acute myelocytic leukemia
ALL = acute lymphocytic leukemia
CML = chronic myelocytic leukemia
BM = bone marrow
PBL = peripheral blood lymphocytes

GM-CSF = Granulocyte-Macrophage colony stimulating factor
IL-3 = Interleukin 3
GCT-CM = Giant Cell Tumor conditioned medium
MLA-CM = a Gibbon T-cell line conditioned medium
IFN = Interferon-gamma
TPA = 12-O-tetradecanoylphorbol-13-acetate
PHA = Phytohemagglutinin
blank = no additions

The results given in this Table indicate that at the end of the culture period in almost every case the total number of cells was lower than at the beginning. The loss of cells during culture can be explained by cell death and adherence to the culture bottle. Although the net cell production is often negative, dividing cells are present in most of the cultures as is reflected by the mitotic index after three days (Table 2.2).

Table 2.2 Mitotic index of the various cultures of leukemia samples

	AML			ALL			CML		
	n	mi	stdev	n	mi	stdev	n	mi	stdev
GM-CSF	6	2.0	1.2						
IL-3+ GM-CSF	14	2.0	2.1	5	1.3	1.5	8	3.4	3.3
GCT-CM	14	3.6	3.1				5	6.4	6.6
MLA-CM	9	1.8	1.7	1	0.6	-	1	2.0	-
MLA+GCT	9	3.3	2.8				2	6.3	1.3
MLA-CM+PHA				2	12.0	10.0			
IFN +TPA				1	0	-			
PHA	14	5.1	5.6	5	3.1	4.2	14	4.4	4.3
PHA enriched	6	7.3	3.9						
blank	12	1.6	1.9	3	2.2	2.2	3	1.6	1.2

Abbreviations as in Table 2.1

mi = mitotic index (number of metaphases/ 100 nuclei)

enriched = T-cell rich fraction from the SRBC rosetting assay

On average the specifically stimulated samples had a higher mitotic index than the controls (blank). Stimulation aiming at specific proliferation of leukemic cells resulted in mitotic indices that were highest in AML and CML when GCT-CM was added; for ALL this was the case with the addition of MLA-CM + PHA. With respect to the number of samples that was tested and the observed standard deviations, the data must be interpreted with care. Chromosomal aberrations were found in none of the ALL cases when processed by flow karyotyping. Therefore, the high mitotic index of ALL cells stimulated with MLA-CM + PHA most likely reflects proliferation of remaining normal lymphocytes.

T-cells were depleted from the bone marrow samples by sheep red blood cell (SRBC) rosetting. In some cases cells could be recovered from SRBC rosettes. These were regarded as enriched T-cell fractions. These cells were also stimulated using PHA (indicated in the table as PHA enriched).

2.1.3 Mitotic arrest

The second requirement is to 'catch' as many cells as possible in mitosis by the use of a spindle poison. Treatment of the cells with a mitotic spindle poison will result in a blockade in the assembly of the microtubuli, the main component for the formation of the spindle apparatus. When spindles are not formed, the chromatids cannot be separated and cells remain in metaphase. The chromatin remains condensed and visible as separate chromosomes in the cells. Isolation of the mitotic cells and release

of the chromosomes in suspension for the purpose of flow karyotyping is discussed in detail in following sections.

In this thesis Colcemid or Tubulazole-C were used as spindle poisons in a concentration of 250 ng/ml for 4 or 16 hours. Theoretically a long blockade (16 hours or longer) by a spindle poison will result in an accumulation of a higher number of mitotic cells than a short blockade (2-4 hours). However, prolonged incubation with a spindle poison could lead to the negative side effect of chromatid separation which is particularly unfavorable for flow karyotyping. This will be discussed in section 2.1.7

2.1.4 Mitotic enrichment by gradient centrifugation for flow karyotyping

From cells that do proliferate only poorly, a sufficient number of mitotic cells can only be obtained when the initial number of cells that was set up in culture was high enough. If such cells grow attached to the surface of the culture dish the mitotic cells can be selectively detached by shaking off. Culturing sufficient numbers of cells will yield a sufficient number of almost pure mitotic cells. Problems arise when mitotic cells cannot be obtained selectively from a culture (e.g. cells growing in suspension). Upon lysis of the cells, only the few mitotic cells will deliver chromosomes in suspension, while all cells will produce particles (organelles, membrane fragments or nuclei). This will unfavorably influence the chromosome/debris ratio. Additional steps have to be taken to improve the quality of the final flow karyogram.

To enhance the final concentration of mitotic cells it was investigated whether mitotic cells could be separated from their interphase counterparts on the basis of their density. For this purpose the in vitro growing BNML cell line LT-12 was used. This cell line grows in vitro in suspension, and it yields sufficient numbers of mitotic cells.

LT-12 cells were set up in culture and after 3 days the spindle poison Tubulazole-C (Janssen Pharmaceutics, Beerse, Belgium) was added at a final concentration of 250 ng per ml. The cells were allowed to accumulate in mitosis for an additional 4 hours after which they were harvested and processed for density gradient centrifugation. The desired gradient densities were obtained by dissolving Nycodenz (Nyegaard, Oslo, Norway) in Hanks HEPES Buffered Balanced Salt Solution (HHBBSS) with 1% bovine serum albumin (BSA). The pH was adjusted to 6.8 and the osmolarity was brought to 280 mOsm. All solutions were checked for their correct density on a digital densitometer (DMA 40; Mettler/Paar, Graz, Austria) at 4 C. Discontinuous gradients were composed in 4 ml plastic tubes (Falcon 2058) by layering 1 ml per density on top of each other. The cell suspension was layered on top of this gradient and spun down at 700 G at 4 C for 20 minutes. The various fractions were collected and washed twice with cold HHBBSS (4 C). The mitotic index and the cell number of

the fractions was determined. Mitotic indices indicate that in this in vitro growing cell line it was possible to selectively isolate the mitotic cells from the interphase cells on the basis of their density (Table 2.3).

The first column shows the densities of which the gradient was composed. The second column shows the mitotic index of the cell samples recovered from the various densities. Mitotic LT-12 cells have a lower density as compared to interphase cells. The highest percentage of mitotic cells was found in the fractions obtained from 1.050 and 1.060. From the total cell sample 64% could be recovered from the various fractions. The material recovered from the bottom of the centrifuge tube consisted of debris and dead cells. Proper cell counting could not be performed on this material. A slightl increase in density of 1.062 was employed throughout the rest of the experiments described in this thesis

Table 2.3 Mitotic enrichment of LT-12 cells on a discontinuous density gradient

density	mitotic index (%)	% of total cells
before	12	100
1.050	98	1
1.060	94	9
1.070	10	22
1.080	1	32
bottom	-	-

density: in g/cm^3
before = cells before gradient centrifugation
bottom = cells on the bottom of the tube after gradient centrifugation

An increased mitotic index by density gradient centrifugation is not the only reason for an improvement of the quality of the flow karyogram. Dead cells do not sustain the pressure evoked by the centrifugal force and will sediment on the bottom of the tube together with debris particles. Figure 2.2 shows for the LT-12 cells how the suspension can be monitored before and after centrifugation on a discontinuous density gradient. It shows cells before density gradient centrifugation (a), and after gradient centrifugation on densities 1.062 (b) and 1.080 (c). Panel d shows the material that was recovered from the bottom of the centrifuge tube.

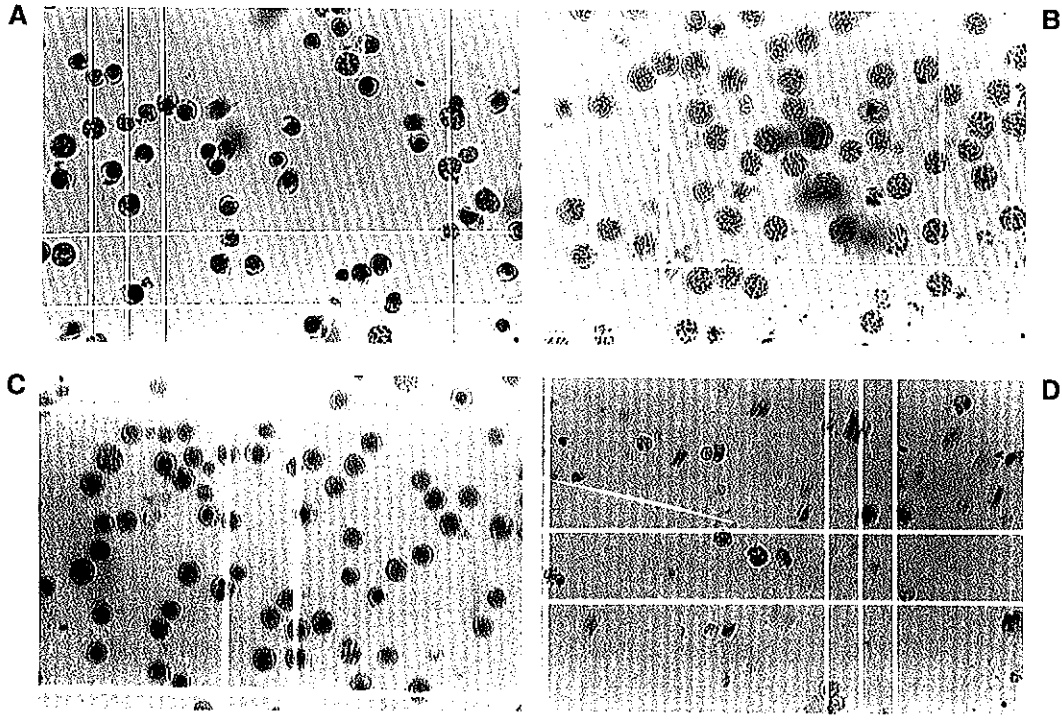

Figure 2.2
Determination of the mitotic index. Cells were recovered from a discontinuous density gradient.
Panel A) Cell sample before gradient
Panel B) Cells recovered from fraction 1.062
Panel C) Cells recovered from fraction 1.080
Panel D) Cells from the bottom to the tube
Cells are stained with Türk's solution and observed in a Bürker type hemocytometer at a magnification of 40X.

The mitotic enrichment of human leukemia cells after gradient centrifugation was determined by counting mitotic cells in each layer of the gradient. Table 2.4 is an extension of Table 2.2 and shows the mitotic index on each density.

Table 2.4 Mitotic index of the various cultures of human leukemia samples at the densities 1.062 and 1.080 g/cm^3 after gradient centrifugation.

		AML			ALL			CML		
	Density	n	mi	stdev	n	mi	stdev	n	mi	stdev
GM-CSF	before	6	**2.0**	1.2						
	1.062	6	**1.8**	1.3						
	1.080	6	**0.4**	0.6						
IL-3+ GM-CSF	before	14	**2.0**	2.1	5	**1.3**	1.5	8	**3.4**	3.3
	1.062	14	**5.1**	5.3	5	**1.5**	2.0	8	**10.8**	16.4
	1.080	14	**0.7**	1.1	5	**1.8**	2.2	8	**2.0**	2.3
GCT-CM	before	14	**3.6**	3.1				5	**6.4**	6.6
	1.062	14	**5.9**	4.0				5	**8.3**	7.0
	1.080	14	**1.3**	4.0				5	**1.0**	1.6
MLA-CM	before	9	**1.8**	1.7	1	**0.6**	-	1	**2.0**	-
	1.062	9	**4.2**	2.7	1	**5.5**	-	1	**11.0**	-
	1.080	9	**1.3**	1.4	1	**1.7**	-	1	**0**	-
MLA-CM+GCT	before	9	**3.3**	2.8				2	**6.3**	1.3
	1.062	9	**5.8**	5.1				2	**48.0**	49.5
	1.080	9	**1.3**	1.7				2	**0.6**	0.9
MLA-CM+PHA	before				2	**12.0**	10			
	1.062				2	**12.1**	12.7			
	1.080				2	**3.1**	1.7			
IFN +TPA	before				1	**0**	-			
	1.062				1	**0**	-			
	1.080				1	**0**	-			
PHA	before	14	**5.1**	5.6	5	**3.1**	4.2	14	**4.4**	4.3
	1.062	9	**4.8**	5.1	5	**2.5**	3.0	14	**4.5**	6.9
	1.080	9	**1.3**	1.7	5	**4.9**	7.0	14	**3.0**	3.5
PHA enriched	before	6	**7.3**	3.9						
	1.062	6	**5.7**	6.2						
	1.080	6	**5.7**	5.1						
blank	before	12	**1.6**	1.9	3	**2.2**	2.2	3	**1.6**	1.2
	1.062	12	**7.2**	16.8	3	**3.8**	3.5	3	**8.1**	13.1
	1.080	12	**1.0**	1.0	3	**0.2**	0.4	3	**1.0**	1.8

abbreviations as in table 2.2
density in g/cm^3

Two densities were chosen (i.e. 1.062 and 1.080) on the basis of the results obtained with the rat leukemia model. It has to be stressed that the various types of human leukemias (and most probably even each individual leukemia) have different densities on which mitotic cells settle. To investigate the optimal density for each

sample would have been too time-consuming and would result in an unfavorable distribution of one limited bone marrow sample over too many density fractions. The rat densities were taken as a general guideline.

2.1.5 Chromosome isolation

Cells in log phase were blocked in mitosis with Tubulazole-C or Colcemid (Calbiochem, San Diego, Ca) as described earlier. The suspensions were washed twice in HHBBSS. From the suspension 25 µl was taken and mixed with 25 µl of water and placed at 37°C. After 5 minutes of swelling, the sample was mixed with 50 µl of Türk's solution and counted in a Bürker type hemocytometer. In this way interphase and mitotic cells could easily be discriminated and the mitotic index determined (Figure 2.2).

To increase the percentage of mitotic cells and removal of dead cells and cell debris, cell suspensions were processed by discontinuous density gradient centrifugation as described above.

The chromosome isolation method described by Bijman (Bijman 1983) was modified to obtain high resolution flow karyograms in clinical samples after the discontinuous density gradient centrifugation. Cells from each separate fraction were centrifuged once more in HHBBSS, the supernatant discarded and the inside of the tube dried as much as possible with a paper tissue. The cell pellets from the fractions were resuspended in chromosome isolation buffer consisting of 20mM NaCl, 8mM $MgCl_2$ and 20mM Tris-HCl pH 7.5 and placed at 37°C for 5 minutes. The swollen cells were lysed by addition of an equal volume of a Triton X-100 solution (0.8% in water) to the chromosome isolation buffer. Subsequently chromosomes were released in suspension by careful shearing through the tip of an Eppendorf pipette. The lysed suspensions were centrifuged for 3 minutes at 150 G. In this way a large fraction of the nuclei could be removed. The supernatant which contains the chromosomes was transferred to a new tube.

Chromosomes were stained at least 2 hours prior to measurements with Propidium Iodide (PI) (10 µg/ml) for univariate measurements or with Hoechst 33258 (Ho) DAPI (5.4 µM) and chromomycin A3 (CA3) (26 µM) for bivariate analysis on a dual-laser beam flow cytometer. In most cases chromosome suspensions were stored at -20°C after the addition of DMSO (10% final concentration). Frozen chromosome suspensions could be stored for weeks, defrosted and refrozen again without visible loss of resolution in the flow karyogram.

2.1.6 Instrumentation for flow cytometry

Uni- and bivariate analyses were performed using the RELACS III, a flow cytometer specially designed and built at the Radiobiological institute of TNO, Rijswijk, The

Netherlands, to measure chromosomes in suspension (Stokdijk et al. 1985, Van den Engh and Stokdijk 1989). The chromosome suspension was introduced into the cuvette by the use of a perfusion pump that was modified to hold the 1 ml syringes that contain the chromosome suspensions. The pump enabled careful control of the rate of injection of the chromosome suspension in between 1.5 to 29.0 µl per minute into the core of the sheath fluid stream.

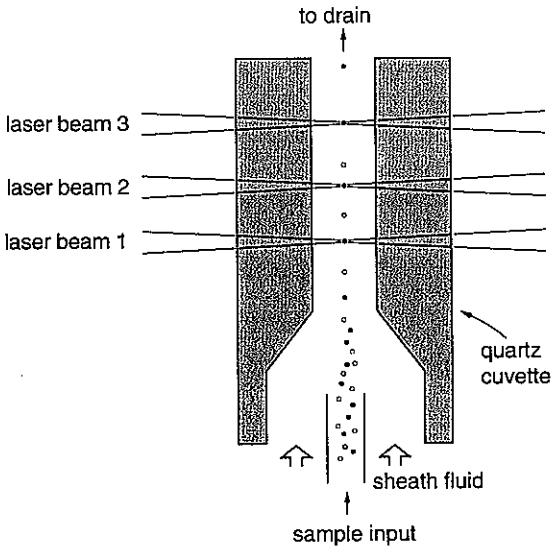

Figure 2. 3
Schematic representation of the RELACS III quartz cuvette. From the three lasers laser 1 and 2 were used for chromosome measurements.

The fluid and sample streams move upwards instead of downwards. Hydrodynamic focusing of the chromosome sample is ensured by the special shape of the quartz cuvette (Figure 2.3). Filtered water (Milli-Q; Millipore, Molsheim, France) was used as sheath fluid. The throughput of the sheath fluid (rate between 8 to 12 ml per minute) was controlled by a peristaltic pump which ensured a minimum of fluctuation and enabled careful and reproducible adjustment so that optimal hydrodynamic focusing of the chromosome suspensions at the laser-interception points was achieved.

The sample was usually run through the flow cytometer at a rate ranging from 20 to 800 chromosomes per second, depending on the concentration of chromosomes in the suspension.

The RELACS III was designed to operate with 1 to 3 lasers (Figure 2.4). For the purpose of bivariate analysis of chromosome suspensions two lasers were directed on the cuvette with two spherical lenses which can be moved independently. The two lasers intersect the cuvette at approximately 200 µm distance from each other. The capacity to move the lens in three dimensions enabled one to steer the laser

beam and to focus it on the cuvette at the measuring point. The lens units create oval shaped spots at the point where the chromosomes are measured. Signals generated at the measuring point in the cuvette are also spatially separated by a set of pinholes behind the cuvette with a diameter of 1 mm. Mirrors are placed at different heights to reflect the light signals of the lasers separately as is shown in Figure 2.4. Up to seven photomultipliers can be used to measure the signals. Due to the special optical design, the RELACS III is a very sensitive flow cytometer with high resolution. Therefore, the machine is very suitable for measurements of chromosomes and determination of flow karyograms. The electronic and data processing parts of the machine are described in detail by Van den Engh and Stokdijk (Van den Engh and Stokdijk 1989).

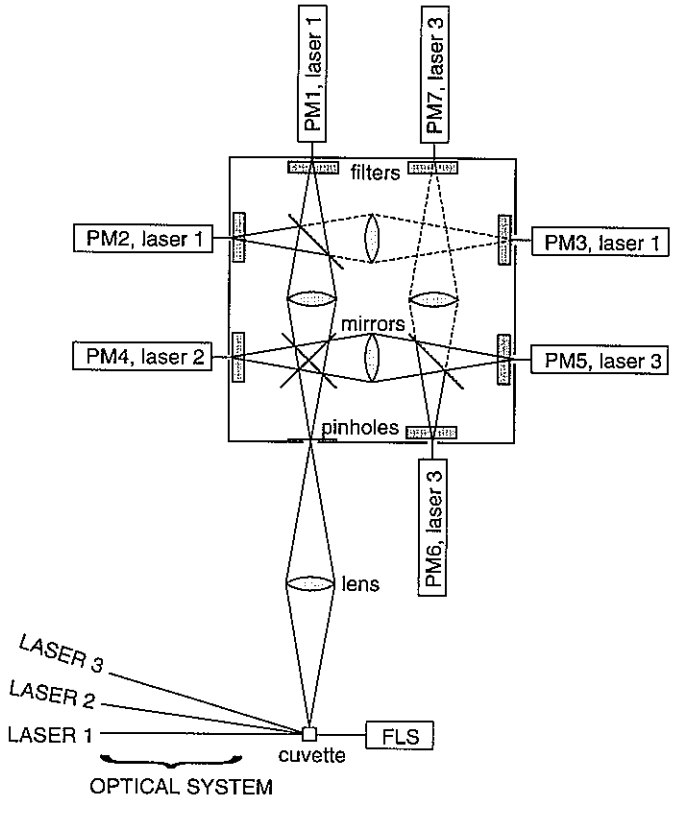

Figure 2.4
Simplified schematic representation of the RELACS III lay-out (top view). For bivariate chromosome measurements two lasers were used.

PM =Photomultiplier
FLS =Forward light scatter detector

PI was excited with an argon ion laser tuned at 488 nm, 500 mW (laser: Coherent Innova 90, Palo Alto, CA, USA). PI fluorescence was measured through two KV 550 filters (Schott, Glaswerke, Mainz, Ger.). Ho or DAPI was excited with an argon ion laser tuned in the UV range (351 nm and 364 nm; laser: Spectra Physics, Series 2000,

Mountain View, CA, USA) at 350 mW laser power. Ho or DAPI fluorescence was measured using two KV 408 filters (Schott). Chromomycin-A3 (CA3) was excited at 458 nm (laser: Coherent Innova 90) at 200 mW. CA3 fluorescence was measured through two 550 nm longpass filters (LL550; Corion Corp., Holliston, Ma., USA).

The fluorescence signal generated by the first laser was used to trigger the electronic pulse processing system. Chromosome measurements were performed by analysis of either pulse heights or pulse integration. Data of approximately 20,000 chromosomes were stored in list mode and subsequently analyzed using ELDAS software (R.R. Jonker and L. Budel; unpublished). For univariate analysis at least 3 parameters were stored (i.e. fluorescence, forward light scatter and time of flight). Bivariate analysis requires that at least 4 parameters are stored. The analysis of relative peak content (i.e. the calculation of the number of events within a cluster) was performed with rectangular windows around the area of interest. Only the events in the window were taken into account ('simple boxing').

2.2 On the presence of separate chromatids in chromosome suspensions

At several occasions during flow karyotyping, a clustering pattern was observed that could not be explained by the presence of chromosomal aberrations. This phenomenon was observed to varying degrees in a number of clinical leukemia specimens. It also appeared to be present in the in vitro growing LT-12 rat leukemia cell line (derived from the BNML) which is employed for preclinical model studies of human acute myelocytic leukemia (Hagenbeek and Van Bekkum 1977, Van Bekkum and Hagenbeek 1977). The BNML as well as the LT-12 cell lines are cytogenetically well defined and the bivariate flow karyogram is thoroughly studied (Arkesteijn et al. 1987). In an attempt to find an explanation, in this section this phenomenon is studied in detail.

2.2.1 Materials and methods

Cell cultures

Cultures of the in vitro established BNML cell line LT-12 were set up at a concentration of 5 x 10^5 cells/ml. Cells were grown in alpha MEM supplemented with penicillin and streptomycin and 5% of FCS. Cell cultures were placed in a humidified incubator which was constantly gassed with 5% CO_2/95% air. In the phase of exponential growth (after 3 to 4 days) cell cultures were treated with Colcemid or Tubulazole-C in various concentrations (ranging from 50 to 500 ng/ml) and incubation times (4 and 16 hours).

Nucleated peripheral blood cells from patients with leukemia were cultured similarly for 4 days at a concentration of 2 x 10^6 cells/ml in the presence of 10% (v/v) of conditioned medium containing GM-CSF.

Preparation of chromosome suspensions for flow karyotyping

Chromosome suspensions were prepared as described in section 2.1.5. In brief, after the mitotic blocking period the mitotic cell fraction was enriched using a discontinuous density gradient as described in section 2.1.4 on all samples. After a swelling period of 10 minutes at 37°C in a buffer containing Mg^{++} ions (8mM), NaCl (20mM) and Tris-HCl (20mM) cells were lysed using an equal volume of Triton X-100 solution (0.8% in water). Chromosomes were stained directly after lysis with Hoechst 33258 (Ho; 5.4 μM) and chromomycin A3 (CA3; 26 μM) at least 2 hours prior to measurements for analysis on a dual-laser beam flow cytometer.

Flow karyotyping

Chromosome suspensions were measured on a dual-laser beam flow cytometer as described in section 2.1.6 The bivariate cluster patterns were compared to the standard average configuration obtained from cell cultures blocked for 4 hours with Tubulazole-C, to detect disappearing or newly arising clusters. Quantitative analysis was performed by calculating the number of events per cluster in each flow karyogram with the aid of ELDAS software by means of 'simple boxing' with square windows.

2.2.2 Results

Figure 2.5 shows the bivariate distribution of human chromosomes from a male CML patient. Mitotic cells were obtained after incubation of the cells for 4 hours with 250 ng/ml Tubulazole-C. As a result of the translocation t(9;22) 2 clusters appear in the karyogram that are not present in karyograms from normal human cells (compare with Figure 1.7). The appearance of a bivariate flow karyogram from CML patients is discussed in detail in Chapter IV.

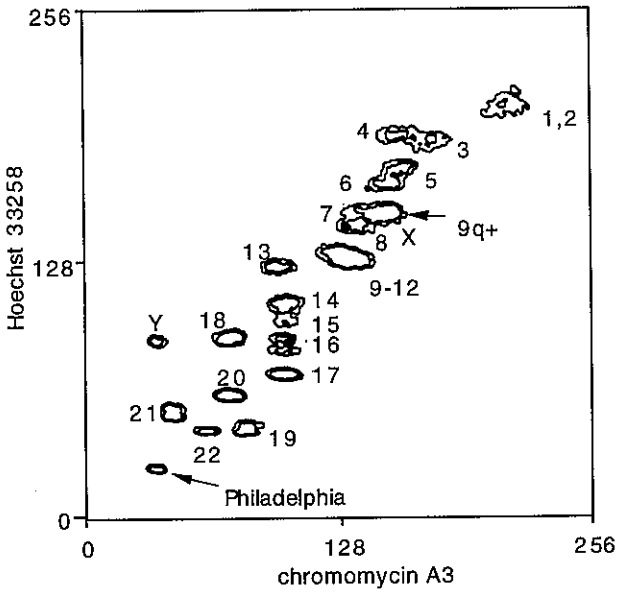

Figure 2.5
Bivariate distribution of chromosomes isolated from peripheral blood cells of a male CML patient. The aberrant clusters caused by the t(9;22) translocation are indicated by arrows.

This bivariate flow karyogram was included for reasons of comparison with the one that is shown in Figure 2.6. In the last figure, the bivariate distribution is shown from chromosomes derived from cultured peripheral blood cells of another CML patient. To obtain chromosomes, the cells from this patient were incubated with 250 ng/ml Tubulazole-C for a prolonged period of time, i.e. 16 hours.

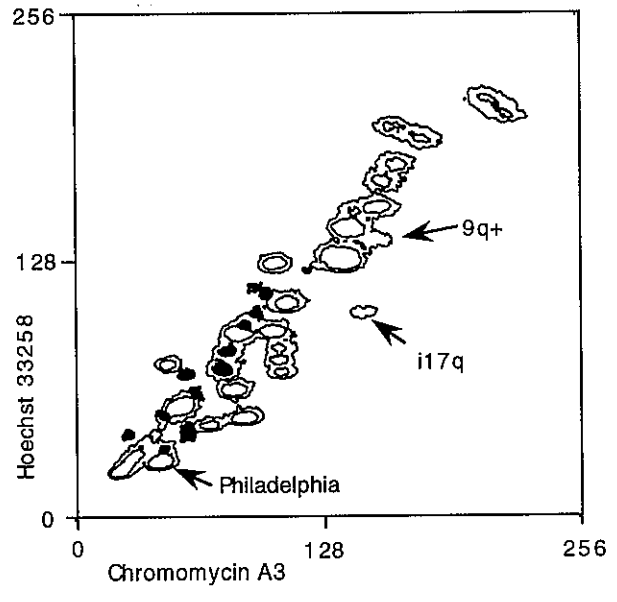

Figure 2.6
Bivariate distribution of chromosomes isolated from peripheral blood cells of a male CML patient. In addition to the known aberrant cluster pattern caused by the t(9;22) translocation and an iso-17q which was also found, a number of aberrant clusters can be observed; indicated in black. To display the aberrant clustering pattern (black) the first contour line was drawn at a lower level than in figure 2.5. Therefore a higher proportion of the debris is visualized at the lower left corner of the karyogram.

The t(9;22) translocation products were observed in their expected position (indicated as Philadelphia and 9q+). Furthermore, as confirmed by conventional cytogenetic analysis a cluster was found that represents an iso-17q. Besides that, clusters appeared in the flow karyogram at unusual places (indicated in black). The relative frequency of these unexpected clusters was determined and appeared to be lower than that of the other chromosomes. The conventional cytogenetic analysis of this patient did not confirm the presence of marker chromosomes that could lead to this unusual clustering pattern.

In Figure 2.7, panel I, a bivariate flow karyogram is shown for the in vitro growing cell line obtained from the BNML. These cells were incubated with Tubulazole-C for 4 hours. To serve as a landmark, some chromosome clusters are indicated as A, B, C, D and E. In panel II a bivariate flow karyogram is shown from the same cell line incubated with Tubulazole-C for 16 hours. The amplifiers were adjusted to show only the small chromosomes. The indications A, B, C and D represent the same clusters as in panel I. Again, in panel II the irregular clustering pattern (in comparison with the regular pattern in panel I) is indicated in solid black. Apparently, the clusters in black (indicated with a, b, c, d and e) contain half the amount of fluorescence and the distribution appears to be similar to the bivariate distribution of the standard pattern as in Figure 2.7 panel I.

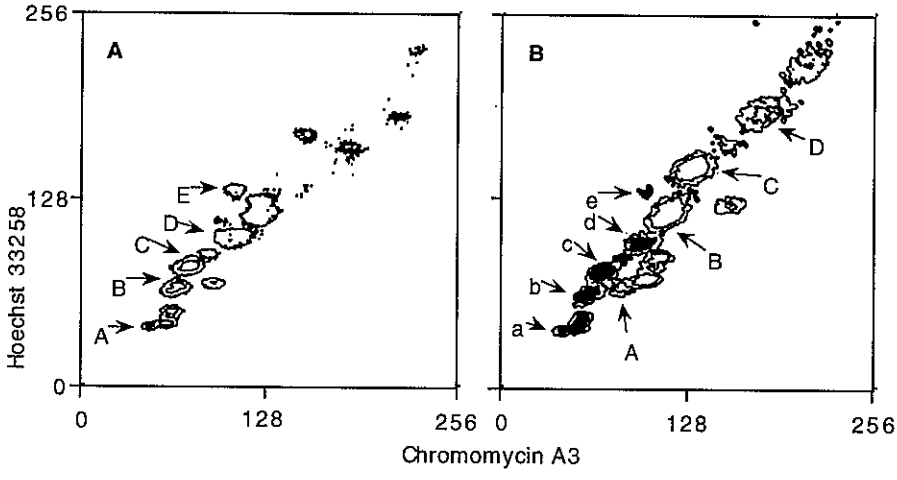

Figure 2.7
Bivariate flow karyograms from the BNML in vitro cell line.
Panel I. Cells incubated with Tubulazole-C for 4 hours .
Panel II. Cells incubated with Tubulazole-C for 16 hours. As compared to panel I, amplifier gains were set approximately twice as high to enhance the small chromosomes. The unexpected clusters indicated in black (a-e), apparently contain half the fluorescence of the flow karyogram of chromosomes. Chromosome clusters A, B, C, and D are corresponding in both panels.

To explain this phenomenon, the contribution of the time of mitotic inhibition as well as the concentration of the spindle poison were investigated. In Table 2.4. the results are given from one representative experiment.

Table 2.4 Relative frequency of unexpected clusters in chromosome suspensions of the LT-12 cell line

	Tubulazole-C		Colcemid	
Incubation time (hrs)	4	16	4	16
Concentration*				
50	nd	nd	10.1%	nd
100	3.5%	12.9%	7.8%	36.2%
250	5.3%	11.6%	24.4%	35.5%
500	8.3%	24.8%	15.4%	42.4%

nd = not determined.
*: concentration in ng/ml; incubation time in hours.

Various concentrations and incubation times of the spindle poisons were tested in the LT-12 cell line. The presence of the aberrant clustering pattern was calculated by counting the number of events found in chromosome cluster E (panel I) and compared to the number of events in the corresponding cluster e (panel II). The presence of events occurring in the background explains the low percentages of events in Table 2.4 when low concentrations of spindle poisons and short incubation times are used. From the table it can be seen that the frequency of aberrant signals increases with both concentration and incubation time. Comparing the effects of Colcemid to those of Tubulazole-C, it seems that at an equal drug concentration the Tubulazole-C effects are less pronounced.

2.2.3 Discussion

Besides the clearly visible Philadelphia chromosome, many additional clusters are found at unexpected positions in the flow karyogram of the CML patient (Figure 2.7, panel II). The pattern formed by these clusters (indicated in black) matches the normal distribution rather well, albeit at half the fluorescence intensity for both fluorescence parameters.

A possible explanation is that the aberrant clustering pattern is caused by the presence of single chromatids in the chromosome suspension, originating from chromosomes that are separated at the centromere. This normally occurs during the anaphase of the mitotic cell division. Routinely the cell cultures were blocked in mitosis for 16 hours. The number of mitotic cells will increase with time finally yielding larger quantities of chromosomes. This prolongation of the incubation time is accompanied by a higher degree of chromosome condensation. Although this would be unacceptable for the microphotography-based cytogenetic analysis (due to

loss of banding resolution) it is not a particular problem for flow karyotyping purposes. However, from the data of the BNML in vitro cell line it can be concluded that longer mitotic blocking periods and an increase in the concentration of the blocking agent go together with an increase in chromatid segregation (Table 2.4). This phenomenon has been observed to different degrees a number of times in human flow karyograms. For example the flow karyogram from the CML patient in Figure 2.5 does not show a chromatid cluster pattern at all. There is no indication that some chromosomes more frequently divide into single chromatids than others. The effect seems to be random, i.e. when single chromatids were observed in the flow karyogram, they always gave rise to a complete "chromatid flow karyogram".

To exclude that electronic or other machine disturbance caused the shadow pattern in the bivariate flow karyograms, samples were run on different machines. The results were similar. Samples with and without aberrant clusters were observed when run shortly after each other

In the literature chromatid segregation in metaphases has been described as "premature centromere division" or "C-anaphase" by several groups (Mehes 1978, Chamla et al. 1980, Mehes and Bajnoczky 1981, Rudd et al. 1983, Gabarron et al. 1986, Madan et al. 1987, Chamla 1988). Whether the phenomenon described in these references is caused by the same underlying process as the presence of chromatids in chromosome suspensions remains unclear. The presence of chromatids in chromosome suspensions was later confirmed using PCR on flow-sorted chromatids (Telenius et al. 1993). A suspension with both single chromatids and chromosomes might very well lead to misinterpretation in the chromosome analysis and it will certainly yield impurities when chromosomes are sorted. To avoid this problem one could either attempt to obtain a 100% pure chromosome suspension or, conversely, induce 100% chromatid separation. The fact that a high mitotic yield due to a long incubation time (16 hrs) leads to chromatid segregation, might make it attractive to induce this phenomenon in 100% of the mitotic cells. In our experiments, however, it was impossible to reach this goal. Suspensions always contained both chromatids and chromosomes or chromosomes alone. Longer incubation times (24 hours) lead to flow karyograms of lesser quality. Forceful shearing of the chromosome suspension had no effect on the generation of extra chromatids and only random chromosome breaks were observed as reflected by an increase in the background debris level (data not shown).

Incubation of rat cell suspensions for 4 hours with Tubulazole-C at a concentration of 100 ng/ml always results in flow karyograms in which no chromatid clusters are observed. The value of 3.5% in this situation (Table 2.4) reflects the background level. However, the presence of chromatids cannot be excluded.

The data in Table 2.4 indicate that chromatid segregation is also influenced by increasing the dose of the spindle poison with a fixed time of inhibition or by

increased duration of the mitotic inhibition with a fixed dose of spindle poison. So far, it can be concluded that it is advisable not to extend the spindle poison blocking period beyond 4 hours. Under such conditions chromatids are not observed with either Colcemid or Tubulazole-C. It has to be stressed, however, that small amounts of single chromatids might still be present which remain unnoticed by regular analysis of the flow karyogram. Their number may be too small to detect them as separate clusters. The presence of small amounts of chromatids might still play a role in sort impurities. For the purpose of probe generation by PCR of sorted chromosome fractions, cross hybridization might occur between probes generated from, for example, sorted chromosome 14 and chromosome 3, or probes generated from probe 20 with 7 and X, since these chromosomes overlap the positions of the chromatid clusters (Figure 2.6).

2.3 Interphase cytogenetics with fluorescence in situ hybridization

In this thesis FISH was used as a procedure to follow-up patients with leukemia. Patients with numerical chromosomal aberrations in their leukemic cells were selected, as judged by conventional cytogenetic analysis that were followed from the time of diagnosis, through the phase of complete remission (detection of "minimal residual disease") to relapse, if this occurred. Furthermore, we followed patients who underwent sex-mismatched allogeneic BMT (SMM allo-BMT) on the basis of the presence or absence of donor- or recipient sex chromosomes to determine the degree of chimerism. All patient studies were performed on bone marrow samples.

2.3.1 Labeling of probe DNA

The probes that are used in this thesis are repetitive sequences specific for mainly alpha satellite DNA sequences on unique chromosome pairs. Probe DNA was inserted into plasmids and used to transform E. coli bacteria that were grown in cultures. The plasmids were isolated and checked for the presence of the correct insert by restriction enzyme digestion and DNA gel electrophoresis. Intact plasmid as well as purified insert were labeled and used as DNA probe. Complete plasmid with insert was labeled with either biotin-16-dUTP or digoxigenin-11-dUTP using the nick-translation procedure (Sambrook et al. 1989). The efficiency of the labeling procedure was checked by spotting samples onto nitrocellulose membranes for the amount of incorporated biotin and by DNA gel electrophoresis for the length of the fragments. Average probe fragment sizes of 200 to 400 base pairs allowed hybridization patterns in which chromosome specific fluorescent spots could be seen in interphase.

2.3.2 Fluorescence in situ hybridization

Cell suspensions were treated with hypotonic buffer (75 mM KCl) for 10 minutes at room temperature. Cells were spun down and fixed with Carnoy s fixative under

continuous vortexing. This procedure was repeated 3 times. Fixed cells were used either directly or were stored in fixative at -20°C for later analysis. After fixation, cells were spotted onto cleaned microscope slides and the area was marked with a diamond tipped pen.

Directly before the hybridization procedure slides were placed in ethanol for at least 60 minutes at room temperature and air dried. Slides were then incubated in 0.1 M HCl/0.1% Tween-20 for 15 minutes to remove matrix and histon proteins and to improve accessibility of the nuclei for the DNA probe.

Denaturation of the target DNA was accomplished by placing the slides in 70% formamide/2x standard saline citrate(SSC) pH 7 at 70°C for 2 minutes followed by dehydration in an ice-cold ethanol series of 70%, 85% and 100%. The hybridization mixture consisted of 1 µg/ml probe DNA, 1 mg/ml sonicated herring sperm DNA, 0.1% Tween-20, 10% dextran sulfate and 2 x SSC in 50% formamide at pH 7. The probe was denatured in the hybridization mixture for 10 minutes at 90°C and quenched on ice immediately. Approximately 12 µl of this denatured hybridization mixture was pipetted onto the marked area of the microscope slide. The area was covered with a plastic coverslip and the slide was placed in a humid atmosphere at 37°C. Hybridization was performed for 4 hours. After hybridization slides were washed 3 times for 2 minutes in 2 x SSC followed by a stringent washing in 50% formamide, 2 x SSC. Five minutes at 45°C was used routinely but with probes known for high non-specific binding, stringency was increased by either increase of the temperature, of the formamide concentration or of the time of washing. Slides were washed in 2 x SSC and finally placed in 4 x SSC/ 0.05% Triton X-100 (SSC-T).

Visualization of the biotin-labeled probe binding was accomplished by incubation of the slides with avidin-FITC (av-FITC) conjugate; 5 µg/ml (Vector, Burlingame, CA, USA) in 5% non-fat dry milk in SSC-T with 0.002% sodium-azide, for 20 minutes at 37°C. After this period, cells were washed 3 times for 2 minutes each with SSC-T buffer. In those cases where amplification of the signal was required, slides were incubated with biotin-labeled goat-anti-avidin; 5 µg/ml (Vector) in 5% non-fat dry milk, for 20 minutes at 37°C. After washing, the av-FITC incubation step was repeated. When single hybridizations were performed nuclear DNA was counterstained with propidium-iodide (1 µg/ml) in Slowfade (Molecular Probes, Eugene, OR, USA). When double hybridizations were performed the biotin-labeled probes were detected using Avidin-Texas red (Vector) (red fluorescence) and the digoxigenin labeled probes were detected using anti-digoxigenin-FITC (green fluorescence) while DNA was counterstained with DAPI (1µg/ml).

Figure 2.8 shows what can be expected when cells and/or metaphase spreads are processed for FISH with one chromosome specific probe.

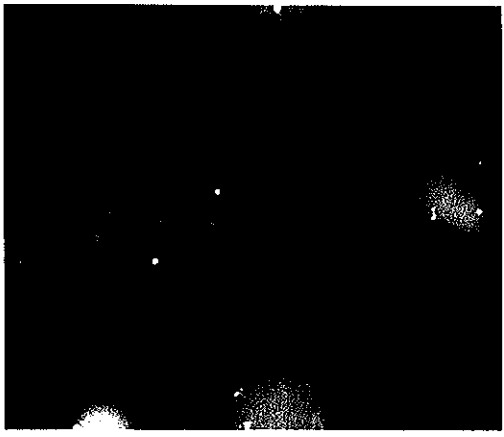

Figure 2.8
FISH on a metaphase slide from a healthy individual. Hybridization with the chromosome 12 specific DNA probe shows two fluorescent signals in metaphase and interphase cells.

In samples obtained from healthy individuals a number of cells can be observed in which the hybridization pattern does not reflect the diploid number of chromosomes that can be expected in these cells. The phenomenon of false positive and negative hybridization is discussed in detail in Chapter VI.