

Chapter VII

**Hybridization of nuclei in suspension with
chromosome specific DNA probes for the
application of flow cytometric detection of
chimerism in sex-mismatched bone marrow
transplanted patients and for the detection of
leukemia after sorting**

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7.1 Summary

Flow cytometry was used to measure fluorescence intensity of nuclei that were subjected to fluorescence *in situ* hybridization in suspension with chromosome specific DNA probes. Paraformaldehyde-fixed nuclei were protein digested with trypsin and hybridized simultaneously with a biotin- and DIG-labeled chromosome specific centromere probe. A number of probes was tested in the suspension hybridizations. The method applied, yielded fluorescent hybridization signals that allow discrimination between Y chromosome positive and negative nuclei when analyzed by flow cytometry. The method is especially suited for analysis of bone marrow cells derived from patients who have received a sex-mismatched allogeneic bone marrow transplantation. Male leukemic cells with a trisomy for chromosome 8 were mixed with normal female cells and simultaneously hybridized in suspension with a DIG-labeled probe specific for chromosome 8 and the biotin-labeled Y chromosome probe. Y chromosome positive or negative nuclei were sorted onto microscope slides and subsequently classified as being leukemic or not by fluorescence microscopy, on the basis of the presence of a trisomy for chromosome 8. A 120-fold enrichment could be achieved when 300 Y positive nuclei were sorted from a mixture originally containing 0.5% leukemic cells. Given the specificity of the flow cytometry and FISH procedure, the combination of the two methods can reach a lower detection level of 1 per 250.000

7.2 Introduction

Fluorescence *in situ* hybridization (FISH) with chromosome specific repetitive DNA probes can be used to identify numerical chromosome aberrations in cells (Devilee et al. 1988b, Hopman et al. 1988, Van Dekken et al. 1990b, Jenkins et al. 1992). In contrast to conventional cytogenetics, where metaphases are required, this interphase cytogenetic technique allows rapid analysis of a great number of cells.

The lower level of detection of FISH has been reported in literature to range from 1% to 5%, depending on the probe and the type of aberration (Poddighe et al. 1991, Chen et al. 1992). This level is set by the occurrence of nuclei with an aberrant number of spots in samples from healthy individuals. Further reduction of the level of detection would require additional independent tumor associated parameters together with the possibility to quantitatively enrich for specific subpopulations and the possibility to analyze a higher number of cells.

The combination of FISH and flow sorting would fulfill these requirements. A large number of cells can be measured in flow cytometry and sorting provides a means to selectively enrich for suspected cells. Hybridization of cells or nuclei in suspension is a prerequisite. FISH on cells or nuclei in suspension has been used for the study of the nuclear architecture by confocal laser scan microscopy (Trask et al. 1988, Van Dekken et

al. 1990d, van Dekken et al. 1990, Hulspas and Bauman 1992, Van Dekken and Hulspas 1993) but found only limited use for magnetic separation of chromosomes (Dudin et al. 1987, Dudin et al. 1988) and measurements by flow cytometry (Trask et al. 1985, Trask et al. 1988, Van Dekken et al. 1990a). In situ hybridization of cells or nuclei in suspension followed by flow cytometry can only be performed provided that the nuclei remain intact after hybridization. Because fixation of nuclei and the stringency of the in situ hybridization procedure negatively influence each other, a balance must be found between these procedures. Nuclei might be well hybridized but not suited for adequate flow cytometric measurement because positive and negative populations cannot be discriminated due to an increased coefficient of variation or vice versa. In this study, a procedure is described by which nuclei retain enough of their shape to allow in situ hybridization in suspension and subsequent flow cytometric analysis of the hybridization signal.

It was found that the difference in fluorescence signal between nuclei with and nuclei without a Y chromosome is large enough to discriminate between positive and negative clusters when nuclei were hybridized with a biotin-labeled Y chromosome specific probe that was detected with Streptavidin-phycoerythrin (PE). Low numbers of host cells, that are frequently observed in the bone marrow in SMM-BMT patients, need to be further classified to be either normal host cells that survived marrow ablative therapy or residual leukemic cells. The technique described here, allows the study of chimerism in leukemia patients who received a sex-mismatched allogeneic bone marrow transplantation (SMM-BMT). With the combination of FISH and flow sorting, nuclei can be characterized as being leukemic or not. A more than a 1000-fold improvement of the lower detection level for residual leukemic cells is achieved compared to the sensitivity of the separate methods.

7.3 Materials and methods

Preparation of the nuclei

Bone marrow samples were diluted two to four times their volume with Hanks HEPES Buffered Balanced Salt Solution (HHBBSS), layered on top of 15 ml lymphocyte separation medium (LSM; Organon, Durham, NC) with a density of 1.077 g/cm³ and centrifuged at 700 G during 20 minutes. The nucleated cells on the interface layer were collected and washed twice with HHBBSS and finally resuspended in buffer consisting of 20mM NaCl, 8mM MgCl₂ and 20mM Tris-HCl pH 7.5. After 5 minutes of incubation at 37 C the cells were lysed by addition of an equal volume of a Triton-x-100 solution (0.8% in water). Subsequently nuclei were released in suspension by careful shearing through the tip of an Eppendorf pipette.

In situ hybridization in suspension.

The nuclei were washed once more in the same buffer, fixed by adding an equal volume of paraformaldehyde solution (4% in PBS) for 16 hours at 4°C, centrifuged and resuspended in PBS afterwards. Approximately 10⁶ fixed nuclei were centrifuged, treated by protein digestion with trypsin (0.25% in PBS) for 10 minutes at room temperature, washed with PBS and subsequently resuspended in hybridization mixture consisting of 1 mg/ml sonicated herring sperm DNA, 0.1% Tween-20, 10% dextran sulfate and 2 x SSC in 50% formamide. Denaturation of the nuclei was accomplished by incubating at 85°C for 20 minutes under continuous shaking (Eppendorf Thermomixer, Hamburg, Germany). Biotin and the DIG-labeled chromosome specific probes (400 ng each) were denatured simultaneously in hybridization mixture for 10 minutes at 95°C. The denatured probes were added to the nuclei and allowed to hybridize for 4 hours at 37°C under continuous shaking. Probes that were used in the various experiments were repetitive DNA sequences specific for chromosome 1(Cooke 1979), 3(Waye and Willard 1989), 7(Waye et al. 1987b), 8(Donlon et al. 1986), 11(Waye et al. 1987a), 17(Waye and Willard 1986), 20(Waye and Willard 1989), X(Willard et al. 1983) and Y (Amersham, Buckinghamshire, UK.).

After hybridization nuclei were washed in 2 x SSC, centrifuged and resuspended in 50% formamide/2 x SSC at room temperature for 5 minutes. After washing in 2 x SSC, nuclei were resuspended in 4 x SSC with 0.05% triton (SSC-T), containing 5% non-fat dry milk and 10 µg/ml streptavidin-PE conjugate (Vector, Burlingame, Ca) for 1 hour at 37°C. After this period nuclei were washed once with SSC-T and resuspended in PBS. Finally nuclear DNA was stained with DAPI (Sigma, St. Louis, Mi) at 1 µM final concentration. For visual inspection prior to flow cytometry, a small sample was stained with avidin-FITC (Vector, Burlingame, Ca) and propidium iodide instead and analyzed by fluorescence microscopy. After sorting on slides nuclei were allowed to attach to the glass surface by drying. Slides were then placed in EtOH for at least 1 hour. When necessary, a stringent wash was performed to remove aspecific binding of the DIG-labeled probe. Anti-DIG-FITC conjugate (Boehringer, Mannheim, Germany) was used to visualize the DIG-labeled probe hybridization.

In situ hybridization on slides.

The procedure for in situ hybridization was a modified procedure originally described by Pinkel et al. (Pinkel et al. 1986). In brief, cells were fixed with methanol/acetic acid, spotted on clean microscope slides and placed in ethanol for one hour. Probes were labeled with biotin by nick translation. Probe and target DNA were denatured and allowed to hybridize for 4 hours. Hybridization was visualized with avidin-FITC. A Zeiss Axioskop-20 microscope was used to screen the slides with a 63 x objective lens. Every nucleus in the field of view was taken into account. Per slide 300 to 500 nuclei were scored.

Flow cytometry

Nuclei were run through a flow cytometer equipped with two lasers (FACS-Vantage, Becton Dickinson, San Jose, Ca). The system was triggered on the forward light scatter signal. PE was excited by 488 nm argon ion laser light at 100 mW laser power (laser: Coherent Innova 90, Palo Alto, Ca). PE fluorescence was measured through a 575/26 bandpass filter and logarithmically amplified. 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) at 350 mW laser power. DAPI fluorescence was measured using a KV 408 filter (Schott Glaswerke, Mainz, Germany).

Mixtures were processed in ascending order to minimize the risk of contamination of nuclei from previous samples remaining in the tubing system. After each sample the system was flushed with bleach (10% in water). Data of approximately 20,000 nuclei were stored and subsequently analyzed using the Lysys-II program (Becton Dickinson, San Jose, Ca). In all sorting experiments only clusters representing the G1/G0 nuclei were sorted.

7.4 Results

When diploid cells from healthy individuals were analyzed with FISH, a small percentage of cells was found showing an aberrant chromosome number. Therefore FISH in suspension was compared to FISH on slides with the key question being: are the results obtained by FISH in suspension comparable to the results as obtained by FISH on slides? Nine probes were hybridized on peripheral blood cells from a healthy male individual according to both methods. The number of signals per cell was scored by fluorescence microscopy (Table 7.1). Columns 2 through 11 show the distribution of the number of fluorescent spots for the probe that were used. For each probe tested, few nuclei deviate from the expected diploid number of chromosomes either after hybridization in suspension or after hybridization on slides. Comparing the groups with the expected number of diploid spots (i.e.: Table 7.1; columns 6 and 7 for the autosomes and columns 4 and 5 for the sex chromosomes), it appears that in suspension hybridization results in a significantly higher number of diploid cells (paired t-test).

When nuclei that were hybridized in suspension were analyzed on the flow cytometer it appeared difficult to reproducibly find fluorescence differences between nuclei with 1 and 2 or higher numbers of hybridization targets. In contrast, analysis by flow cytometry of the nuclei hybridized in suspension with the Y probe consistently resulted in signal differences between Y chromosome positive and Y chromosome negative nuclei that showed up as clearly separated clusters in the bivariate dot plot (Figure 7.1). To test the sensitivity of the flow cytometric measurements artificial mixtures were made ranging from 0.1% to 99.9% male in female cells. Nuclei were isolated and hybridized in suspension with a biotin-labeled

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Y chromosome specific probe and a DIG-labeled X chromosome specific probe and stained with streptavidin-PE to measure specific Y probe binding (Figure 7.1).

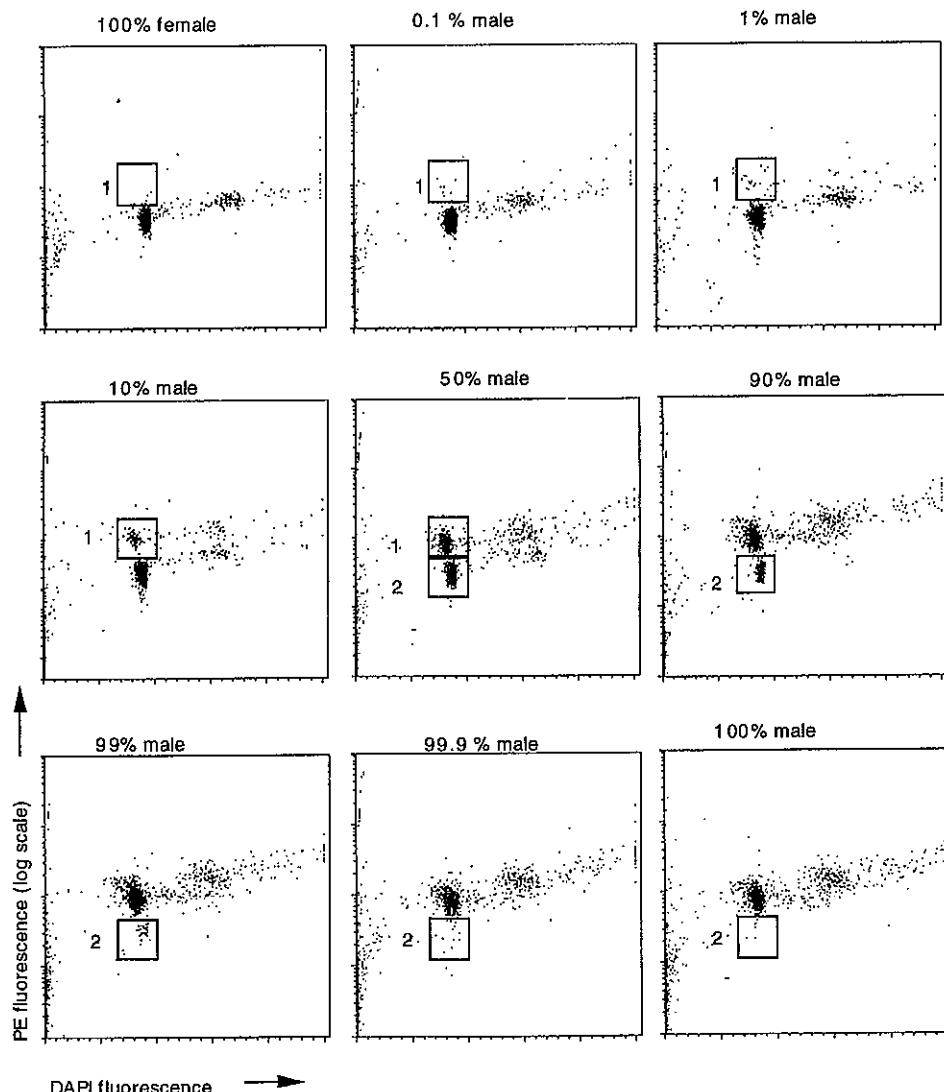


Figure 7.1

Bivariate dot plot of mixtures of male and female nuclei hybridized in suspension with biotin-labeled Y and DIG-labeled X chromosome specific DNA probes. Y probe binding is detected by streptavidin-PE. The dot plots are displayed with DAPI fluorescence (DNA content) on the horizontal axis and PE fluorescence (probe binding) on the vertical axis (logarithmic scale). Sort windows for Y positive and Y negative clusters are indicated as 1 and 2 respectively. The clusters in the area where windows 1 and 2 are set, represent the G1/G0 nuclei. The clusters at twice the DAPI fluorescence represent G2 nuclei and doublets of G1/G0 nuclei.

The percentage of male and female cells that was determined on the basis of flow cytometric analysis of the clusters is given in columns 2 and 3 from Table 7.2. In the mixtures ranging from 1% to 99% male cells, separate clusters could be identified (Figure 7.1). Nuclei were sorted from the regions of interest (indicated as "1" or "2" in Figure 7.1) directly onto microscope slides. The X probe binding was visualized by staining with anti-DIG-FITC conjugate and the nuclei were scored for the presence of one or two fluorescent spots and classified as male or female. Results are shown in Table 7.2.

Table 7.2 Recovery of male or female cells after in situ hybridization in suspension (X and Y probe), flow sorting on the basis of Y probe binding followed by microscopical analysis for chromosome X specific probe binding.

Male cells in mixture	Events in Y positive window	Events in Y negative window	Male cells (1 spot for the X probe) in Y positive window	Female cells (2 spots for the X probe) in Y negative window
0	0.3	99.7	0.7	-
0.1	0.5	99.5	23.2	-
1	1.8	98.2	72.4	-
10	9.8	90.2	94.7	-
50	38.5	61.5	96.0	96.4
90	83.3	16.7	-	98.4
99	97.6	2.4	-	89.1
99.9	99.3	0.7	-	54.9
100	99.5	0.5	-	1.0

all figures in percentages

- = not determined

From the 50% samples, nuclei could be recovered with a high purity (more than 90%). With decreasing percentages of mixed cells the percentage of nuclei that was found back after sorting gradually dropped. Analysis of the sorted fractions of the lowest percentages of mixed cells showed that the amount of nuclei remains far above the lower detection level of FISH-on-slides alone. For the mixture containing 0.1% male cells sorting of the Y positive population resulted in 23.1% nuclei with one fluorescent spot for the X probe. In the reversed setting (0.1% female cells and sorting of the Y negative population) 54.9% of the nuclei were found with 2 fluorescent spots for the X probe. In the female control samples, 0.3% of the events were found in the Y probe positive area. After sorting of these nuclei it appeared that 0.7% had 1 spot for the X probe. In the male control samples 0.5% of all the events appear in the Y negative area, while 1% of these nuclei contained 2 fluorescent spots for the X probe.

Bone marrow cells were obtained from a female leukemia patient who received a SMM-BMT. Based on morphological criteria this patient was in a leukemia relapse. To investigate the applicability of FISH in suspension and analysis on the flow cytometer in such patients, the sex difference was used to discriminate between cell populations from host or donor origin. Analysis by flow cytometry resulted in two distinct clusters; a Y positive cell population with high PE fluorescence and a Y negative cell population (Figure 7.2).

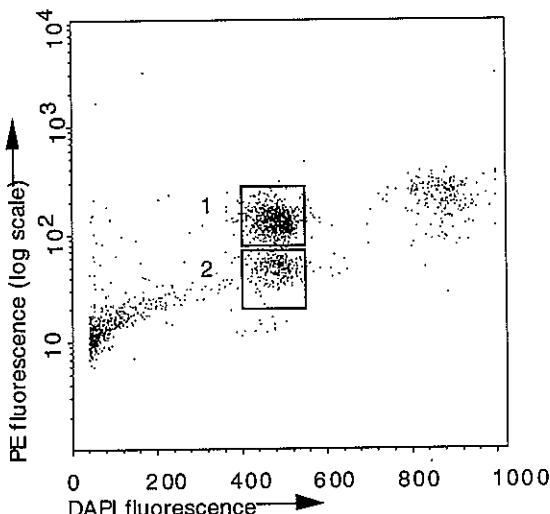


Figure 7.2

Nuclei originating from a female leukemia patient who had received a sex-mismatched bone marrow transplantation and who developed a leukemia relapse. Nuclei were hybridized in suspension with biotin-labeled Y chromosome specific DNA probe. Fluorescence detection of the Y probe binding and axes are as in Figure 7.1. The ratio of Y positive versus Y negative cells was determined by analysis of the number of events in windows 1 and 2.

In the Y positive window (region 1 in Figure 7.2) 10753 events were present and in the Y negative window (region 2 in Figure 7.2) 3793 events. Thus, 26% of the nucleated cells lacked the Y chromosome and were probably of host origin. Residual non-leukemic host cells, however, can be expected in chimeras. This requires an additional leukemia marker to classify these cells as leukemic or not. Therefore, it was investigated whether leukemic cells could be identified after sorting on the basis of the presence of leukemia associated numerical chromosomal aberrations. For this purpose marrow cells from a healthy female were mixed with marrow cells from a male leukemia patient in ratios ranging from 0.5 to 50%. According to conventional cytogenetic analysis, performed on 35 metaphases, 57% of the cells from the bone marrow of the male leukemia patient contained a trisomy for chromosome 8 and an additional 3% carried a tetrasomy 8. With conventional FISH on slides, 70.4% was found to contain three or more spots for chromosome 8 (300 cells were analyzed).

The mixtures were hybridized in suspension with a biotin-labeled Y chromosome specific probe and a DIG-labeled chromosome 8 specific probe and stained with streptavidin-PE to measure specific Y probe binding (Figure 7.3).

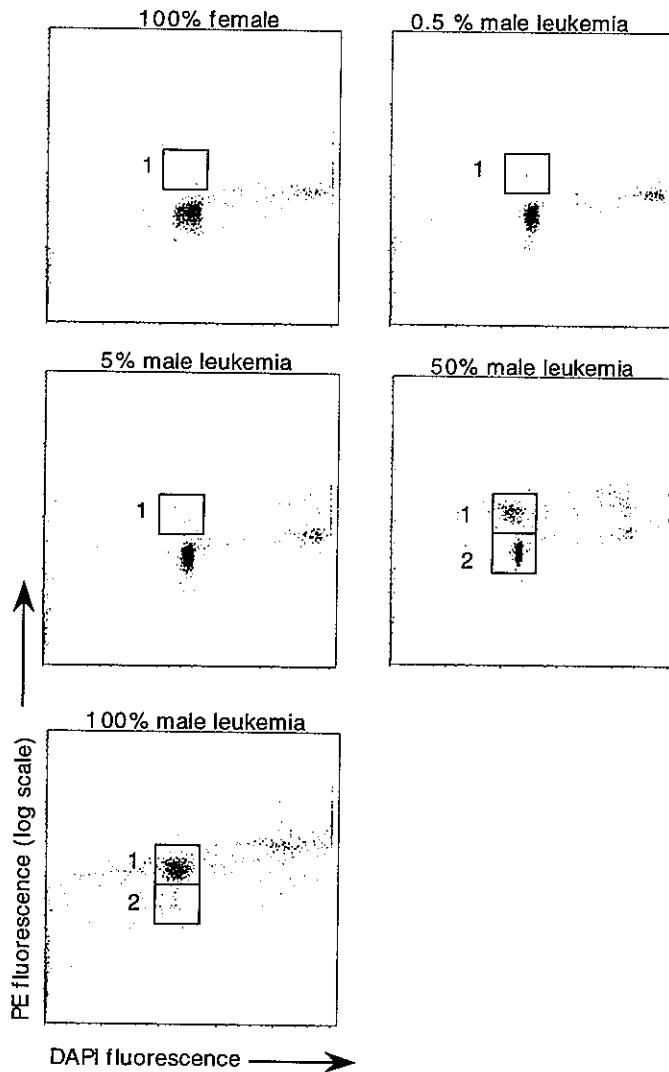


Figure 7.3
 Bivariate dot plot of mixtures of male leukemia and normal female nuclei hybridized in suspension with biotin-labeled Y chromosome specific DNA probe and DIG-labeled probe specific for chromosome 8. Fluorescence detection of the Y probe binding and axes are as in Figure 7.1. Sort windows for Y positive and Y negative clusters are indicated as 1 and 2 respectively.

Y positive and Y negative nuclei were sorted on slides (Figure 7.3; region 1 or 2). After staining of the probe 8 hybridization with Anti-DIG-FITC the presence of numerical aberrations for chromosome 8 was determined with fluorescence microscopy. In some of the sorted fractions the presence or absence of Y probe hybridization was verified by incubation of the sorted fractions on slides with avidin-FITC. The first column in Table 7.3 shows the mixtures that were made.

Table 7.3 Recovery of male leukemic cells after *in situ* hybridization in suspension (8 and Y probe), flow sorting on the basis of Y probe binding followed by microscopical analysis for chromosome 8 specific probe binding

male leukemic cells in mixture (%)	expected cells with 3 or more spots in mixture	sorting of Y positive window		sorting of Y negative window	
		cells in Y positive window (%)	cells with 3 or more spots for probe 8 (%)	cells	cells
				reanalyzed for the presence of 1 Y spot (%)	reanalyzed for the presence of 1 Y spot (%)
0	0	0.1	0.39	0	-
0.5	0.4	0.5	40.9	-	-
5	3.5	1.9	68.4	-	-
50	35.2	29.9	71.3	-	1.7
100	70.4	96.8	68.1	99.0	68.4
					78.0

1: Calculated from conventional FISH on slides (70.4% of all cells have 3 or more spots).

Column 2 shows the expected percentage of nuclei with three or more spots for chromosome 8 in the mixture based on the observation that with FISH on slides 70.4% of the cells in the leukemia sample contained 3 or more spots. In column 3 the percentage of Y positive nuclei is shown as measured by flow cytometry. Column 4 shows the percentage of nuclei with 3 or more spots for chromosome 8 after sorting of the Y positive area. In the fractions sorted from the 5%, 50% and 100% male cell populations, approximately 70% of the nuclei has three or more spots per nucleus for chromosome 8. In the fraction sorted from the 0.5% male cell sample, the percentage of nuclei with 3 or more fluorescent spots for 8 is 40.9. This indicates that a more than 100-fold enrichment is achieved for this fraction. In the sample that contained no male leukemic cells, samples sorted from the Y positive area yielded nuclei with false positive trisomies for chromosome 8 at the background level (0.39%). Evaluation by microscope, indicated that none of the recovered nuclei from this fraction contained a Y chromosome (Table 7.3 column 5). In the Y negative fraction sorted from the 50% sample, 1.7% of the nuclei had 3 or more fluorescent spots for chromosome 8 (Table 7.3; column 6) indicating that a small percentage of trisomy 8 containing leukemic cells lost the Y chromosome. The sample consisting of 100% male cells showed 68.1% and 68.4% nuclei with 3 or more fluorescent spots for chromosome 8 in the Y positive and the Y negative window, respectively. In the same sorted fractions approximately 100% of the nuclei was expected to show one spot for the Y probe when reanalyzed by microscope, albeit present in a very low percentage of the total population in region 2. Although the sort fractions of Y positive area from the 100% male sample contained, as expected, 99% nuclei with 1 spot for the Y probe, the sorted fraction of the Y negative population from this sample, showed that 22% (100% minus 78%) of this sub-population lost the Y

chromosome. The number of events in the Y negative window was 3.2% of the whole population (Table 7.3 column 3; 100% minus 96.8%). Therefore, FISH in suspension combined with flow cytometry and sorting indicates that 0.7% of the total leukemic cell population lost its Y chromosome (i.e. 22% of 3.2%).

7.5 Discussion

The presence of host derived cells in blood and bone marrow from sex-mismatched bone marrow transplanted leukemia patients might be either indicative for mixed chimerism or for the presence of (residual) leukemic cells. The detection of residual leukemic cells with numerical chromosomal aberrations using chromosome specific probes and FISH on slides becomes difficult once the target population drops below 1-3%. In normal cell populations a small percentage of cells is found that shows an aberrant chromosome number when analyzed by FISH (Table 7.1). This phenomenon remains largely unexplained. Either cells contain a real aberrant chromosome number or the hybridization procedure itself might be the cause of erroneous observations. Microscopically based analysis using a combination of immunophenotype and the genotype of a cell was developed (Price et al. 1992). This technique could successfully be employed to monitor the kinetics of mixed chimerism in subpopulations of leucocytes from patients, for the analysis of residual host cells and for the characterization of relapse, at different stages of BMT (Kögler et al. 1995).

Compared to microscopy, flow cytometry allows processing of large numbers of nuclei. Furthermore, nuclei of interest can be sorted and studied for the presence of additional aberrations. Therefore in suspension hybridization was performed to be able to analyze nuclei by flow cytometry. To exclude that the hybridization in suspension as applied in this study yields more nuclei with aberrant chromosome numbers, FISH in suspension was compared to FISH on slides using nuclei from a healthy individual. Hybridization of nuclei in suspension and analysis by fluorescence microscopy did not lead to a greater fraction of aberrant cells than conventional FISH on slides. Though successful in situ hybridization in suspension was achieved for all the probes tested, only hybridization with the Y probe resulted in reproducible fluorescence differences when mixtures of male and female cells were analyzed by flow cytometry. There are several explanations for this. First of all, the Y probe that was used recognizes a large part of the long arm of the Y chromosome resulting in fluorescence intensity that is higher than that observed with the other probes. Furthermore, the use of the Y probe on a mixture of male and female cells shows the difference between 0 or 1 spot. Because of the variation in fluorescence distribution measured on the nuclei with 1, 2, 3 or more spots, it becomes gradually more difficult to detect the difference in fluorescence intensity between 1 and 2, 2 and 3 or more spots, which is required for the detection of numerical aberrations of the other chromosomes. Finally, the use of PE as a fluorescent conjugate appeared to

give more reproducible results for the detection by flow cytometry than FITC conjugated biotin (data not shown).

In the flow cytometric studies of the samples consisting of 100% female, 0.3% of the nuclei appear in the Y positive window. In the 100% male sample 0.5% of the nuclei appear in the Y negative window. Basically these figures reflect the lower detection level for male or female cells in mixtures when flow cytometry is performed alone. The possibility to sort selected subpopulations enables a further reduction of the detection level. Sorting of the Y positive area from the 100% female sample and analysis for X probe hybridization by microscope, shows that 0.7% from this 0.3% (= 0.002%) has one fluorescent spot for the X probe. Sorting of the Y negative area from the 100% male sample shows that 1% of the 0.5% (= 0.005%) contained 2 spots for the X probe.

The ability to measure sex differences between cells is highly relevant for leukemia patients that received a sex-mismatched bone marrow transplantation (SMM-BMT). In Figure 7.2 the use of the Y probe and in suspension hybridization followed by FCM showed a high percentage of host cells. The indication for leukemia relapse was confirmed by morphological criteria. However, low amounts of host derived cells often occur for years in the bone marrow of SMM-BMT patients without clinical signs of relapse. To be able to further characterize these cells as being leukemic or not, the presence of additional leukemia associated markers can be analyzed after sorting. As a model, male leukemic cells with numerical aberrations for chromosome 8 were mixed with normal female cells, hybridized in suspension, analyzed by flow cytometry and sorted. In the 100% female sample 0.1% of the nuclei appeared in the Y positive area. From this fraction 0.39% contained an apparent trisomy for chromosome 8 (Table 7.3). This implies that approximately 1 nucleus per 250,000 (0.39% from 0.1%) would be falsely classified as belonging to the leukemic host cell population. This ratio indicates the lower detection level of the combined approach of flow cytometry and FISH for the experiment as performed in this paper. The following consideration has to be made. When nuclei are sorted at high purity with an efficiency of 95% and approximately 300 nuclei are required on a slide to perform a significant FISH analysis by microscope, it would require at least 316 leukemic cells in the analyzed sample. At a 1 to 250,000 ratio this means that at least 79×10^6 total cells should be processed in one sample. This indicates that the amount of cells that can be processed in one sample is a limiting factor.

The calculations of the ratios of male and female cells by flow cytometry were performed on G1/G0 clusters only (area 1 and 2 in all the dot plot figures). Based on the observations of the dot plots it is highly unlikely that more than 50% of the cells were not in G1/G0. The selection of the G1/G0 will at most lead to an underestimation of a factor of 2.

Several factors are of influence to the successful hybridization in suspension. First of all a proper fixation with 4% paraformaldehyde for 16 hours. Secondly a protein digestion with 0.25% trypsin to detach cell clumps, and thirdly the detection of probe hybridization in flow using PE instead of FITC. FISH in suspension as described in this paper allows clear discrimination between positive and negative nuclei when analyzed by flow cytometry. The additional value of FISH combined with flow cytometry and sorting and its value for detection of residual leukemic cells is demonstrated. Other applications, like non-invasive prenatal diagnosis, are envisaged. Further developments resulting in discrimination of signals with other chromosome specific probes will further expand the area of applications.

