FLUORESCENCE IN SITU HYBRIDIZATION TO ISOLATED CHROMOSOMES IN SUSPENSION

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Table of Contents

page

List of Figures

page

List of Tables

page

INTRODUCTION

Fluorescence *in situ* hybridization (FISH) is a powerful technique that has found a wide range of applications in modern molecular and cell biology. Rapid advances in FISH have allowed its use in areas ranging from gene mapping and prenatal diagnosis, to charaterizations of complex chromosome abnormalities and genome wide DNA alterations associated with various cancers.

DNA-DNA and DNA-RNA denaturation and reannealing properties had been well characterized by the late 1960's (McConaughy *et al* 1969) and these properties were soon manipulated to allow *in situ* hybridizations using radio-labeled RNA probes. The earliest of these *in situ* hybridizations detected abundant sequences on polytene chromosomes of Drosophila (Gall and Pardue, 1969, John *et al* 1969) and on metaphase chromosomes of humans (Evans *et al* 1974). While these isotope labeled probes gave good results, the lengthy time involved, the radiation exposure risk for workers, and the disposal of radioactive waste all combined to make alternative, non-radioactive, labeling methods highly desirable. An important milestone in the realization of non-isotopic *in situ* hybridization was the development of biotin-labeled polynucleotides (Langer *et al* 1981). Research showed that biotinylation of DNA did not

cause an appreciable change in hybridization kinetics and gave researchers the ability to uniquely label DNA sequences without the problems associated with isotope labeled probes. After hybridization, the biotinylated DNA duplex was visualized using avidin, which binds tightly to biotin, followed by an anti-avidin antibody to which a detection molecule had been coupled. The first successful FISH experiments utilizing biotinylated probes were performed on Drosophila polytene chromosomes (Langer-Safer *et al* 1982) and mouse metaphase chromosomes (Hutchison *et al* 1982; Manuelidis *et al* 1982). These initial hybridizations consisted of single probes detected fluorimetrically or cytochemically. The target areas in early FISH work were usually greater than 50 Kb but in a few cases areas as small as 25 Kb could be detected (McNeil *et al* 1991).

Today, FISH work employs a variety of directly labeled, multi-color probes covering areas as small as 2-5 Kb (Trask, 1991) to as large as the entire genome (Pinkel *et al* 1986). The material to which FISH is applicable now includes tissue sections (Brigati *et al* 1983), blastomeres (Grifo *et al* 1992) and first polar bodies (Munné *et al* 1996), sperm cells (Wyrobek *et al* 1990) and interphase cells in suspension (Trask *et al* 1985).

One area where FISH has seen important applications is in the detection of structural chromosome abnormalities. The technique of "chromosome painting", first introduced in the late 1980's (Pinkel *et al* 1986, Lichter *et al* 1988, Pinkel *et al* 1988), allows a single chromosome to be fluorescently labeled along its entire length while the other chromosomes in the metaphase spread are counter stained in a contrasting color. If the labeled chromosome is involved in a translocation with a "non painted" chromosome, the derivative chromosome is easily visualized as a bi-color chromosome. Chromosome painting has been used extensively for the in vitro study of dose-response frequencies upon exposure to ionizing radiation (Lucas *et al* 1989, Cremer *et al* 1990, Natarajan *et al* 1992, Schmid *et al* 1992, Weier *et al* 1991, Lucas *et al* 1992, Bauchinger *et al* 1993) and for testing models of chromosome aberrations (Lucas and Sachs, 1993). Reverse chromosome painting (Carter *et al* 1992), a powerful variation on chromosome painting, allows for the rapid determination of the chromosome of origin, as well as the break-points, of aberrant chromosomes. Forward and reverse chromosome painting have been used in combination to characterize complex karyotypes that where indeterminable using traditional karyotyping (Carter, 1994).

When translocations or inversions involve a known breakpoint, probes can be developed which overlap that breakpoint. After hybridization of the breakpoint spanning probes, inversions or translocations are scored by assessing the location of the hybridization signal on the chromosomes. For an inverted chromosome, two signals will appear on the target chromosome; for translocated chromosomes, a portion of the signal will appear on the target chromosome while the remaining portion of the signal will appear on the derivative chromosome. This method has proven useful for detection of translocations and inversions in interphase nuclei (Tkachuk *et al* 1990, Dauwerse *et al* 1990). More recently, breakpoint spanning probes have been used on blastomeres for pre-implantation genetic diagnosis (PGD) of patients carrying inversions (Cassel *et al* 1997) and translocations (Munné *et al* in press).

Flow sorting of cells based on their DNA content had been developed in 1969 (Hulett *et al*), but was limited by the fact that cells could only be separated if there was a large difference in their total DNA content (e.g. diploid versus tetraploid). In 1975, Grey *et al* reported the characterization of Chinese Hamster metaphase chromosomes by flow cytometry and in 1979 Corrano *et al* reported the same application using human chromosomes. In both methods, DNA

binding dyes were employed which bound to chromosomes and gave off an amount of fluorescence corresponding to the chromosome size. In both studies, however, due to size similarities, chromosomes could only be separated into a limited number of peaks, preventing complete sorting of each of the individual chromosomes. In 1985, Trask et al developed a protocol that allowed hybridization of a mouse satellite DNA probe to interphase nuclei which were not imobilized on a glass slide but were in suspension. With this development, it now became possible to analyze cells based not on total DNA content but based on the presence or absence of specific DNA or RNA sequences (Timm EA Jr & Stewart CC, 1992). If Trask's FISH in suspension could be applied to chromosomes prior to flow sorting, it would allow researchers to uniquely label any chromosome and perform high resolution flow sorting based on that label. The combination of FISH in suspension to isolated chromosomes and flow cytometric analysis would also allow translocation detection to be done more rapidly and reproducibly. Current methods of translocation detection require manual analysis of each individual metaphase, a tedious and time consuming task. Flow cytometry of hybridized chromosomes would allow hundreds of thousands of chromosomes to be examined in a matter of minutes. This would also lower the detection

limit for translocation induction as the number of chromosomes being analyzed could be greatly increased.

This type of translocation detection would be especially applicable to the detection of diseases involving chromosome translocations, such as chronic myelogenous leukemia (CML), in which over 90% of cases are caused by a translocation between chromosomes 9 and 22. Current methods of detecting CML include FISH to blood derived metaphase chromosomes on slides (Tkachuk *et al* 1990, Zhang *et al* 1993), FISH to bone marrow derived metaphase chromosomes on slides (Seong *et al* 1995), PCR on genomic DNA (Miyamura *et al* 1993) and in situ RT-PCR on the BCR/ABL transcript (Testoni *et al* 1996). However, each of these methods requires manual analysis of individual cells or metaphases, thereby limiting the number of cells or metaphases that can be realistically evaluated. For early detection, or detection of minimal residual disease after bone marrow transplants, it is desirable to score a large number of cells; something which could be accomplished utilizing FISH in suspension and flow cytometry.

Because of its potential applications, researchers began attempts to perform FISH to isolated chromosomes in suspension; however, it quickly became apparent that human metaphase chromosomes did not retain their structure during

the harsh conditions required for denaturation (Trask *et al* 1985). A method of encasing chromosomes in "gel microdrops" was developed which mimicked the structural support normally afforded by glass microscope slides and allowed chromosomes to be hybridized in suspension and subsequently used for flow cytometry (Nguyen, B-T *et al* 1995). However, this is a time consuming procedure and has only been successfully reported using mouse chromosomes. Interestingly, human chromosomes obtained from human-hamster hybrid cell lines were able to survive the hybridization in suspension (Dudin *et al* 1987) and be subsequently analyzed using slit scan flow cytometry (Hausmann *et al* 1990). However, this technique does not allow for direct studies of human patients or evaluation of persons exposed to radiation or other translocation inducing agents.

Because of its many potential applications, this study was designed to develop protocols that will allow for the successful hybridization of fluorescent probes to isolated chromosomes in suspension. Because flow cytometric analysis of the hybridized chromosomes is the next logical step in the development of this technique, work was also done to determine the cell culture and chromosomes isolation procedure which give the optimal flow histogram of the isolated chromosomes and to perform the initial flow

cytometric analysis of chromosomes that have been hybridized in suspension.

MATERIALS AND METHODS

ISOLATION AND LABELING OF PROBE DNA

The plasmid pUC1.77, carrying alpha satellite III DNA specific for chromosome 1 (Cooke et al, 1979), was isolated using the QIAGEN Plasmid Prep procedure. This procedure is basically an alkaline lysis procedure as described by Sambrook et al, 1989. Total isolated plasmid DNA was directly labeled in FluoroRed (Amersham, Arlington Heights, IL) or Fluorescein isothyocyanate (FITC) (Boeringer Mannheim, Indianapolis, Indiana) using a standard random priming procedure as follows: Approximately 800 ng of plasmid DNA was combined with water to a final volume of 28 µl, denatured 10 minutes at 95° C, and placed immediately on ice. The following items were then added: 10 µl 10X A4 mix (2 mM each dATP, dGTP, dCTP, 0.1 mM EDTA pH 8.0,10 mM Tris-HCl pH 7.0), 6.5 µl 1 mM dTTP (Boeringer Mannheim), 3.5 µl fluoro labeled dUTP and 20 µl 2.5X random primers (BioPrime kit, Gibco BRL, Gaithesburg, MD). The sample was vortexed well and centrifuged briefly. One microliter of Klenow

fragment (BioPrime Kit, Gibco BRL, 40 Units/µl) was added and the sample was mixed gently and incubated 4 hours at 37° C. After incubation, 10 µl of 10X stop buffer (0.2 M EDTA, BioPrime kit, Gibco BRL) were added and 1 µl of probe was run on a 2% diagnostic gel to verify the reaction had worked.

CELL CULTURE

HSF-7 cells (foreskin strain developed by D. Chen, Los Alamos National Laboratory) were obtained from LLNL frozen stock, thawed quickly in a 37° C water bath, and transferred to a T-25 flask containing 5 ml of media (DMEM/F12, 20% FBS, 1% P/S/G, Gibco, BRL) and incubated overnight at 37° C. The following day the media was aspirated off, the cells were washed one time with PBS, and 1 ml of 0.25% trypsin was added. Cells were trypsinized for approximately two minutes and shaken off by firmly rapping the flask against the palm of the hand. Five milliliters of media were then added to stop the trypsin action and the entire volume was transferred to a T-75 flask containing 9 ml of fresh media. The cells were cultured until they reached approximately 80-90% confluency at which time they were split again. Cells were cultured for two to three passes in the T-75 flasks.

This allowed enrichment for cells that attached less firmly to the substrate and thereby released more easily during the shake off procedure. After two to three passes in the T-75 flasks, the cultures were split into T-150 flasks (two T-150 flasks per T-75 flask) and cultured to 80-90% confluency. The cultures were split as described above into fresh T-150 flasks containing a total of 30 ml of media until there was a total of 10 cultures in T-150 flasks. When these cultures reached approximately 80-90% confluency fresh media containing Colcemid (Gibco/BRL, 10 µg/ml) at 0.1 µg/ml was added and cells were incubated an additional 16 hr at 37° C. Mitotic cells were then shaken off and taken immediately into the chromosome isolation procedure.

GM 130B cells, a lymphoblast derived cell line, (Mutant Cell Repository, Camden, NJ) were obtained from LLNL frozen stocks and thawed as above. The cells were transferred to a 15 ml conical tube containing 9 ml media (RPMI 1640, 15% FBS, 1% p/s/g, Gibco, BRL), centrifuged 5 min at 250 g and the supernatant removed. Cells were resuspended in 10 ml fresh media, counted and plated at a concentration of 4 x 10^5 cells/ml of media, and incubated at 37° C. For chromosome isolation, four T-75 flasks were seeded with 16 X 10^6 cells at 4-5 X 10^5 cells/ml. Cells were allowed to grow for one generation (approximately 24 hrs) at which time colcemid was added at 0.1 µg/ml. Blocking continued for 12-

16 hrs after which cells were centrifuged and the media removed. Cells were then taken immediately into the isolation procedure.

CHROMOSOME ISOLATION

The isolation procedure is adopted from the method described by Blumenthal, et al, 1979.

Isolation Buffer:

15 mM Tris base
2 mM EDTA
0.5 mM EGTA
80 mM KCl
20 mM NaCl
0.2 mM spermine
0.5 mM spermidine
14 mM β-mercaptoethanol
0.1% digitonin

Stock concentrations of the first five ingredients were made up as follows:

I. 150 mM Tris base
800 mM KCL
200 mM NaCl

II. 20 mM EDTA

III. 5 mM EGTA

The isolation buffer is prepared on the day of use as follows: five ml of each of the stock solutions I-III was combined with 35 ml of distilled water and the pH adjusted to 7.2. Fifty microliters of β-mercaptoethanol and 0.06 g of digitonin were added and the buffer incubated at 37° C for 45 min. Twenty five microliters of stock concentrations of spermine (0.4 M) and spermidine (1.0 M) were added and the buffer was filter sterilized.

Mitotic cells obtained from Colcemid treatment were transferred to conical tubes (one 50 ml tube per T-150 or T-75 flask) and centrifuged for 10 min at approximately 250 g. The supernatant was removed and cells were resuspended in 5 ml of hypotonic buffer (75 mM KCl, 0.2 mM spermine, 0.5 mM spermidine) and swelling was allowed to proceed for 25 min at room temperature, after which the cells were centrifuged at 250 g for six min and the supernatant removed. Cells

were then resuspended in 1 ml of chromosome isolation buffer and placed on ice for 10 min. Cells were vortexed vigorously for 30 sec and placed on ice for 5 min. A 10 µl sample was removed, combined with 5 µl of 4,6-diamidino-2-phenylindole (DAPI) (0.25µg/ml, Sigma, St. Louis, MO), and mounted on a slide for viewing under a fluorescence microscope.

PROTEASE INHIBITOR

10X protease inhibitor (cat. # 158837, ICN Biomedicals Inc., Aurora Ohio) was added to the isolation buffer to a final concentration of 1X. The inhibitor consisted of AEBSF (4-(2-Aminoethyl)-benzenesulfonyl fluoride) (250 µg/ml), EDTA-Na (5 µg/ml), Leupeptin (1 µg/ml), and Pepstatin (1 µg/ml). Chromosome morphology was compared visually with and without the protease inhibitor immediately after the hybridization procedure. One hundred chromosomes from each sample were examined for the presence or absence of a recognizable centromere structure.

HYBRIDIZATION

One hundred microliters of isolated chromosomes were aliquoted into a 5 ml Falcon tube (Becton Dickinson,

Franklin Lakes, NJ) and 1 ml of methanol:acetic acid (3:1) was added. Chromosomes were incubated 15 min at room temperature then centrifuged (10 min at approximately 320 g for this and all subsequent chromosome centrifugations) and the supernatant removed. Its is important that all the supernatant is removed to maintain correct pH and formamide concentration in the hybridization buffer. Chromosomes were resuspended in 48.5 µl of dH_2O, 2.5 µl of probe, 109.2 µl of hybridization buffer (20 mls formamide, 2 mls 50X Denardt's Solution, Sigma, 10 mls 20X SSC (Sambrook et al, 1989), 4 mls dH_2O). The sample was then denatured 2.5 min at 73° C and quickly put on ice. Hybridization was allowed to proceed 2.5 hrs at 37° C after which 200 µl of 2X SSC were added to reduce viscosity and the sample was centrifuged and the supernatant removed. Chromosomes were resuspended in 500 µl of wash buffer (0.1 X SSC, 5 mM EDTA, 500 µg/ml BSA, Sigma) and washed 5 min at 37° C. The sample was again centrifuged, and the supernatant removed. For visualization of hybridization results, chromosomes were fixed in 300 µl of fixative for 10 min at room temperature, centrifuged, resuspended in 20 µl of fixative and dropped on a clean glass slide. PI or DAPI and a coverslip were added and the results viewed using fluorescence microscopy.

POLYAMINE STABILIZATION OF CHROMOSOMES DURING HYBRIDIZATION

To prevent degradation of the chromosome structure during hybridization, the polyamines spermine and spermidine were added to the hybridization buffer. Spermine and spermidine were added at concentrations 3.1, 31.2, and 312 µM and 1.24, 12.4, and 124 µM respectively. The hybridizations were then carried out in the absence of probe. One hundred chromosomes per sample were scored for the presence or absence of a recognizable centromere structure following the hybridization procedure and the results compared using a standard T test.

METHANOL:ACETIC ACID FIXATION

Immediately after the chromosome isolation procedure, 20 drops of 3:1 methanol:acetic acid fixative were slowly added to the chromosomes while gently mixing. The chromosomes were then centrifuged and the isolation buffer removed. A total of 1 ml fixative was added drop wise while vortexing the sample on the lowest setting. The sample was then incubated at room temperature for 30 min, centrifuged, and the supernatant removed and replaced with 1 ml of fresh fixative.

FLOW CYTOMETRIC ANALYSIS

Various components of the chromosome isolation procedure were investigated independently for their effect on the resulting flow histogram. Chromosomes were isolated as described above with the particular component being investigated varied as described below. Isolated chromosomes were run on a Becton Dickinson FACScan with the flow histogram divided into seven sections corresponding to various peaks or areas of interest. The number of events and peak height in each area were recorded.

Colcemid Treatment

Colcemid treatments were done as two experiments. The first experiment included times from 9-15 hrs while the second experiment included time from 2-8 hrs. Cells were blocked in mitosis using Colcemid for varying lengths of time. The isolated chromosomes were then run on the FACScan to determine which concentration resulted in the best flow histogram.

Hypotonic Treatment

To determine the optimal time for cell swelling, the length of time the cells remained in the hypotonic treatment was varied and the isolated chromosomes were run on the FACScan to determine which hypotonic time gave the best flow histogram.

Vortexing

To determine the optimal time of vortexing for cell lysis and chromosome dispersal, cells were centrifuged for increasing amounts of time and run on the FACScan to determine which times resulted in the best flow histogram.

Propidium Iodide (PI) Staining

Chromosomes were stained with concentrations of PI ranging from 0.5 µg/ml to 50 µg/ml and run on the FACScan to determine the concentration of PI which resulted in the best flow histogram.

Flow Cytometry of Hybridized Chromosomes

For flow cytometry, chromosomes were isolated using the culturing and isolation conditions determined to be optimal (i.e. 6 hrs colcemid treatment, 20 min hypotonic treatment, 15 sec vortexing, and 15 µg/ml propidium iodide staining). Chromosomes were hybridized with the FITC labeled probe and counter stained with PI.

RESULTS

CHROMOSOME ISOLATION

HSF-7 cells blocked for 16 hrs with colcemid routinely gave isolated chromosomes with good morphology and yield and low debris levels. However, there were a large number of clumps which could not be reduced by further vortexing without damaging the monodispersed chromosomes. GM 130B cells blocked 6 hrs with colcemid routinely gave chromosomes with excellent morphology and yield, minimal clumping, and low levels of debris.

POLYAMINE STABILIZATION OF CHROMOSOMES DURING HYBRIDIZATION

The use of spermine and spermidine, at concentrations of 20 and 50 mM respectively, gave statistically more stable chromosomes for the HSF-7 cell line (i.e. more chromosomes had recognizable centromere structures) (table 1).

Polyamine Concentration	No. of chrom. with centromere	No. of chrom. without centromere	Total chromosomes	p value
none	48	52	100	1.00
0.5 µl/ml	67	33	100	0.08
5.0 µl/ml	66	34	100	0.09
50.0 µl/ml	78	22	100	0.01

Table 1. Chromosome stabilization effects of polyamines. p values for the presence of recognizable centromeres in chromosomes hybridized in the presence and absence of spermine and spermidine.

The stabilizing effects of the polyamines was also tested on the chromosomes obtained from the GM 130B cell line but no difference was seen between chromosomes denatured in the presence of and in the absence of polyamine even at concentrations of 20 mM spermine and 50 mM spermidine (p value = 0.8).

PROTEASE INHIBITOR

Without the use of protease inhibitors, the HSF-7 derived chromosomes completely lost their structure when combined with the hybridization buffer. In the presence of the protease inhibitors, the HSF-7 derived chromosomes maintained their structure in the hybridization buffer and maintained a recognizable structure after short denaturation times. The GM 130B derived chromosome retained their structure without the protease inhibitors when combined with the hybridization buffer and withstood denaturation for up to 5 min. However, after prolonged storage at 4° C the chromosome morphology became progressively worse and by four weeks storage, no recognizable chromosomes were seen after the hybridization procedure. The protease inhibitors allowed the chromosomes to be successfully hybridized after longer storage at 4° C but degradation of the chromosomes continued to occur and stored chromosomes did not have as good a morphology after hybridization as did freshly isolated chromosomes.

METHANOL:ACETIC ACID FIXATION

GM 130 B derived chromosomes that have been fixed retain their morphology after hybridization for up to four weeks after isolation. Multiple fixations breakup the large

pieces of cellular membrane that remain after the isolation but fail to remove it.

FLUORESCENCE IN SITU HYBRIDIZATION

Excellent hybridization signals were routinely obtained using the above protocol on GM 130B derived chromosomes. The signals were bright with minimal or no non-specific hybridization (figure 1). Probe did, however, become trapped in the cytoplasmic/membrane background material remaining in the chromosome suspension. This resulted in a light speckling effect on the background material but had no effect on the ability to detect hybridization signal.

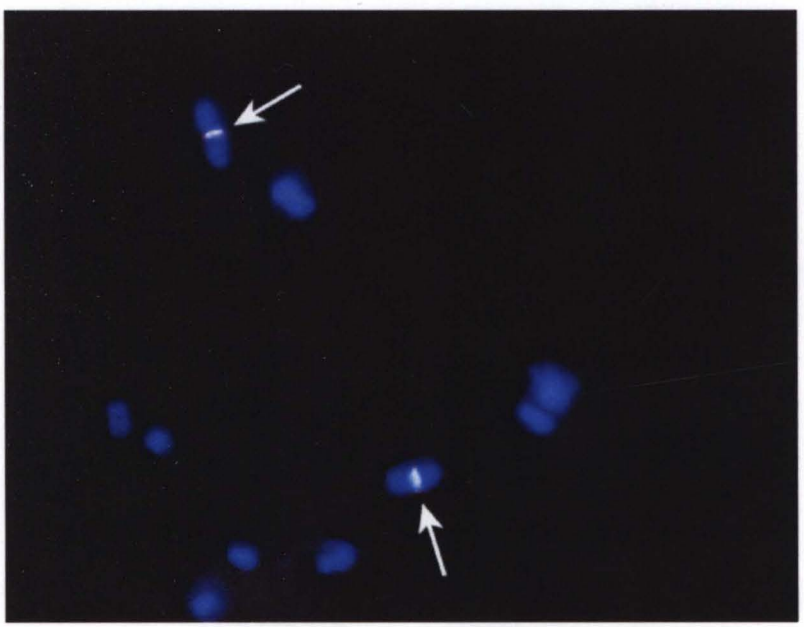

Figure 1. Isolated chromosomes hybridized with SpectrumRed labeled pUC1.77. Arrows indicate hybridization signal.

FLOW CYTOMETRIC ANALYSIS

An optimized flow histogram for human chromosomes stained with propidium iodide is shown in figure 2. Chromosomal and cellular debris normally form a continuum in the first area of the histogram. Therefore, an increase in the number of events occurring in the first area of the flow histogram (relative to the total number of events observed) was taken to represent an increase in the total level of debris.

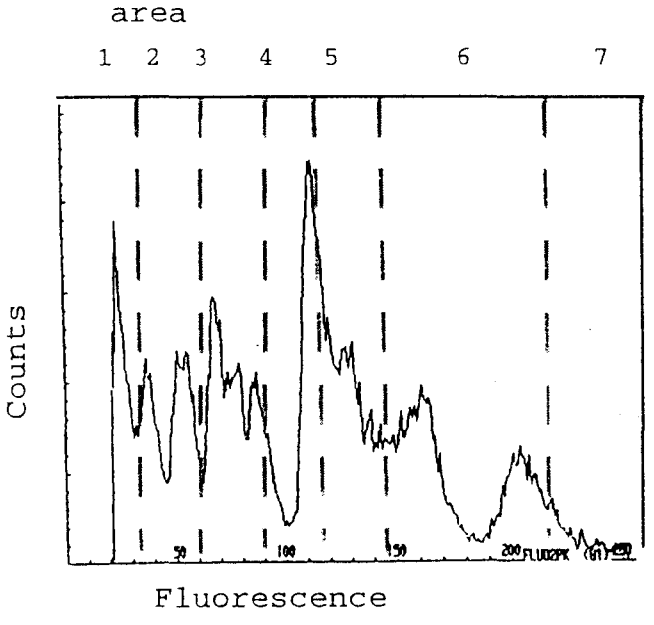

Figure 2. Optimized flow histogram for human chromosomes stained with propidium iodide and run on a flow sorter.

For this study, chromosomes isolated for investigation of particular components of the system were isolated from different cultures. Therefore, a direct comparison cannot be made between chromosomes isolated for investigation of different components (e.g. comparisons cannot be made between the flow histogram obtained from the Colcemid work and the flow histogram obtained from the vortexing work).

Colcemid Treatment

Colcemid treatments were done as two experiments. The first experiment included times from 9-15 hrs (figure 3, table 2) while the second experiment included time from 2-8 hrs (figure 4 and table 3). For both experiments, as the time of Colcemid treatment increased, so did the percentage of events occurring in the first area of the flow histogram. For the 9-15 hr treatments, peak heights and peak distribution remained relatively constant. For the 2-6 hr treatments, 6 hrs treatment gave a flow histogram with the greatest resolution and the largest peak in area seven.

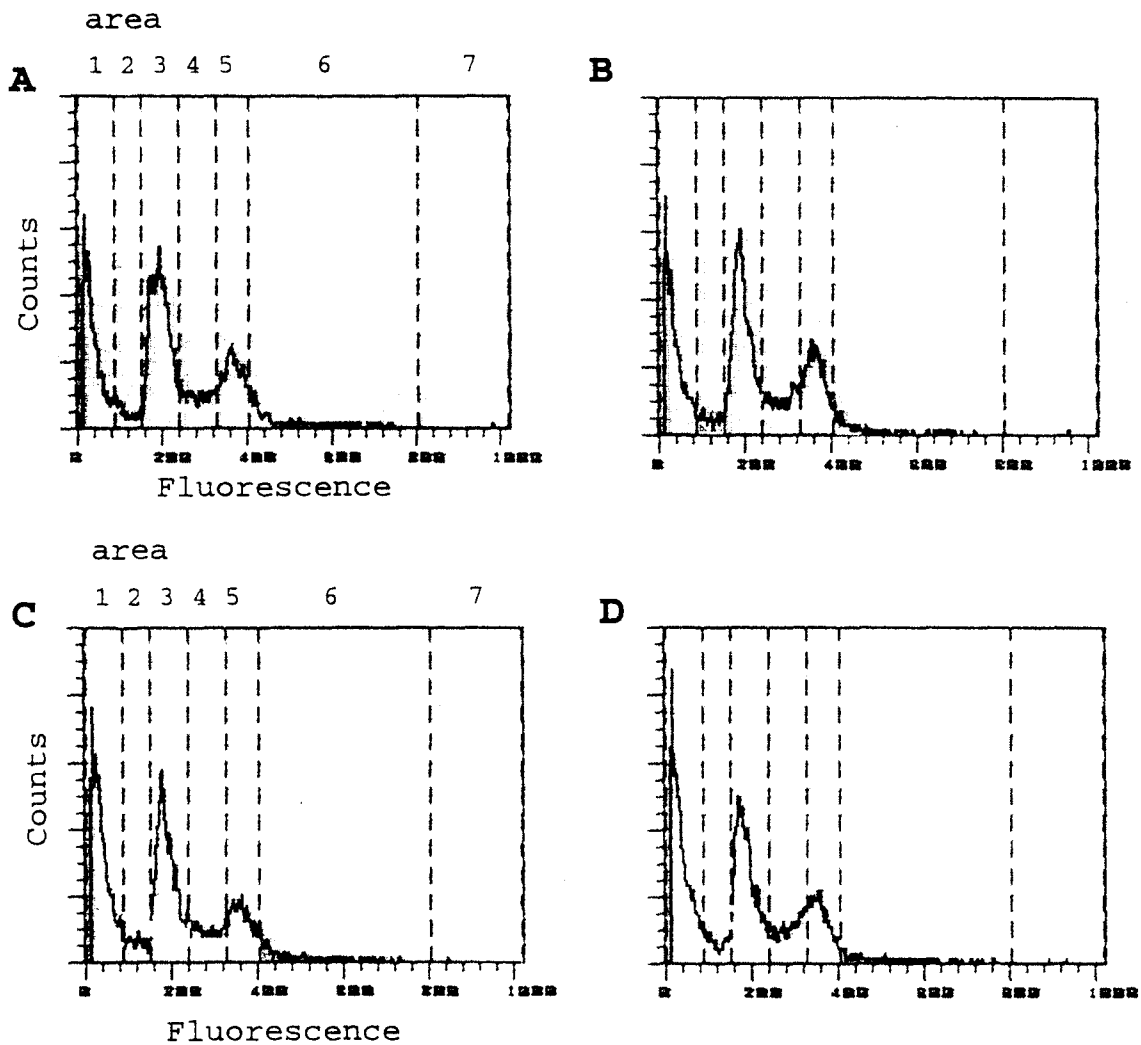

Figure 3. Flow histograms for various lengths of colcemid treatment. A) 9 hrs, B) 11 hrs, C) 13 hrs, D) 15 hrs. Chromosomes were stained with 25 µg/ml PI.

Section	Total	Percent	%CV	Peak Height
1	1168	23.36	51.5	41
2	206	4.12	17.21	7
3	1802	36.04	10.29	35
4	556	11.12	9.33	9
5	838	16.76	5.49	16
6	415	8.3	18.5	8
7	7	0.14	2.06	1

A

Section	Total	Percent	%CV	Peak Height
1	1197	23.36	51.86	45
2	196	4.12	15.94	5
3	1725	36.04	10.08	39
4	611	11.12	9.29	10
5	930	16.76	5.33	18
6	281	8.3	18.37	7
7	3	0.14	0.1	1

B

Section	Total	Percent	%CV	Peak Height
1	1525	30.50	49.53	49
2	252	5.04	150.70	6
3	1632	32.64	11.01	37
4	533	10.66	9.28	10
5	689	13.78	5.59	13
6	317	6.34	16.68	6
7	6	0.12	10.04	1

C

Section #	Total	Percent	%CV	Peak Height
1	1532	30.64	51.70	56
2	280	5.60	16.96	8
3	1550	31.00	11.40	32
4	633	12.66	8.91	11
5	699	13.98	5.40	14
6	271	5.42	17.88	3
7	7	0.14	5.68	1

D

Table 2. Event distributions for flow histograms generated using different lengths of colcemid treatment. A) 9 hrs, B) 11 hrs, C) 13 hrs, D) 15 hrs. Chromosomes were stained with 25 µg/ml PI.

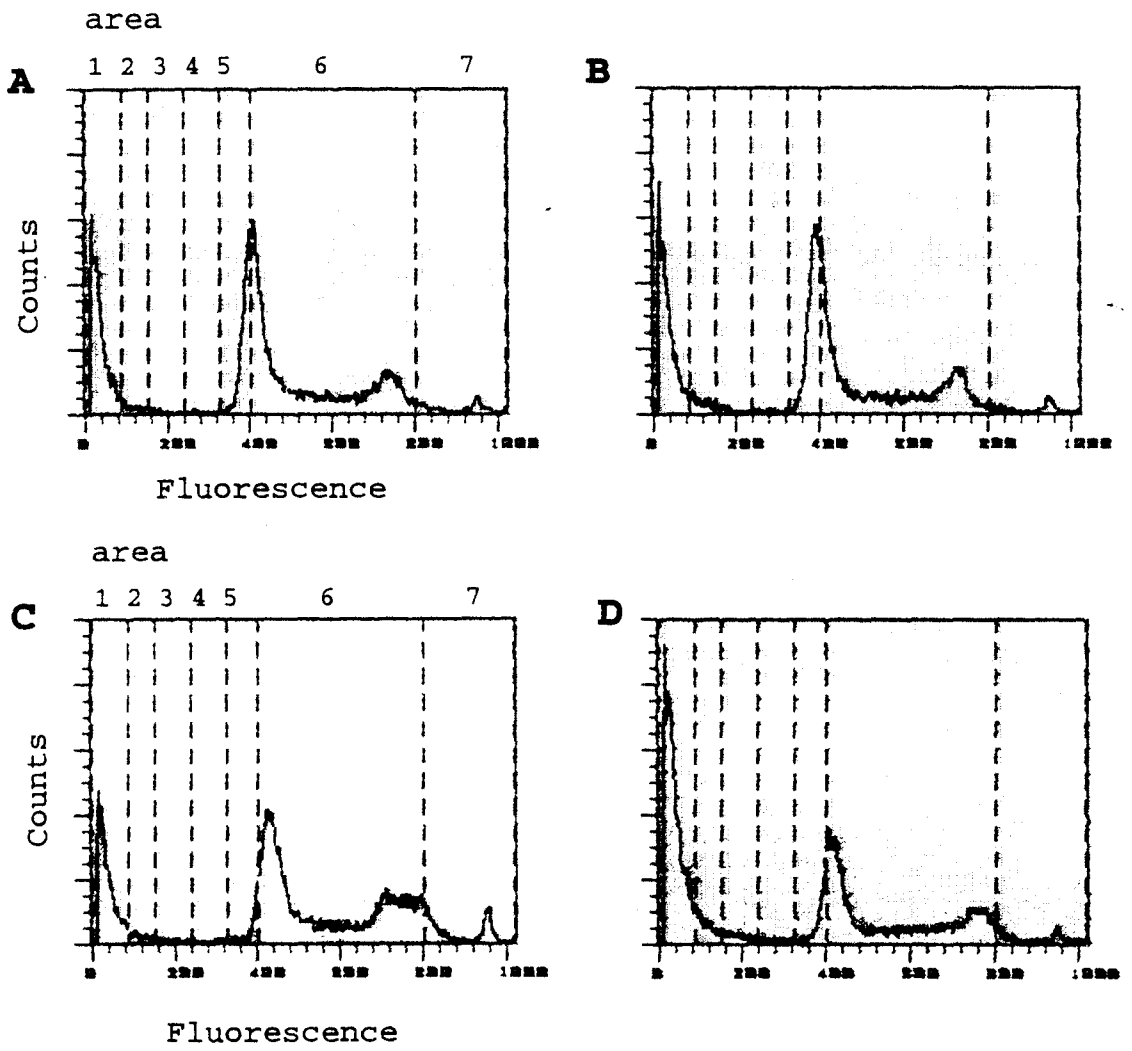

Figure 4. Flow histograms for various lengths of colcemid treatment. A) 2 hrs, B) 4 hrs, C) 6 hrs, D) 8 hrs. Chromosomes were stained with 25 µg/ml PI.

Section	Total	Percent	%CV	Peak Height
1	2227	22.27	50.47	79
2	171	1.71	16.93	6
3	69	0.69	11.26	2
4	73	0.73	8.32	2
5	1559	15.59	3.06	74
6	5438	54.38	24.21	76
7	455	4.55	7.36	9

A

Section	Total	Percent	%CV	Peak Height
1	2398	23.98	50.65	91
2	283	2.83	15.55	8
3	113	1.13	13.09	3
4	113	1.13	9.1	2
5	2286	22.86	3.53	74
6	4426	44.26	23.51	69
7	375	3.75	6.68	7

B

Section	Total	Percent	%CV	Peak Height
1	1802	18.02	50.96	61
2	192	1.92	15.50	5
3	106	1.06	12.24	2
4	100	1	8.60	2
5	401	4.01	4.95	24
6	6505	65.05	23.91	53
7	897	8.97	7.66	15

C

Section	Total	Percent	%CV	Peak Height
1	3850	38.5	49.57	118
2	478	4.78	15.67	12
3	263	2.63	13.16	5
4	140	1.4	8.73	3
5	591	5.91	4.25	34
6	4204	42.04	24.53	46
7	459	4.59	7.70	8

D

Table 3. Event distributions for flow histograms generated using different lengths of colcemid treatment. A) 2 hrs, B) 4 hrs, C) 6 hrs, D) 8 hrs. Chromosomes were stained with 25 µg/ml PI.

Hypotonic Treatment

Flow histograms from the various times of hypotonic treatment are shown in figure 5 and the event distribution is shown in table 4. Times from 20 to 60 min all gave similar flow histograms without appreciable differences in distribution of events, peak heights, or histogram shape.

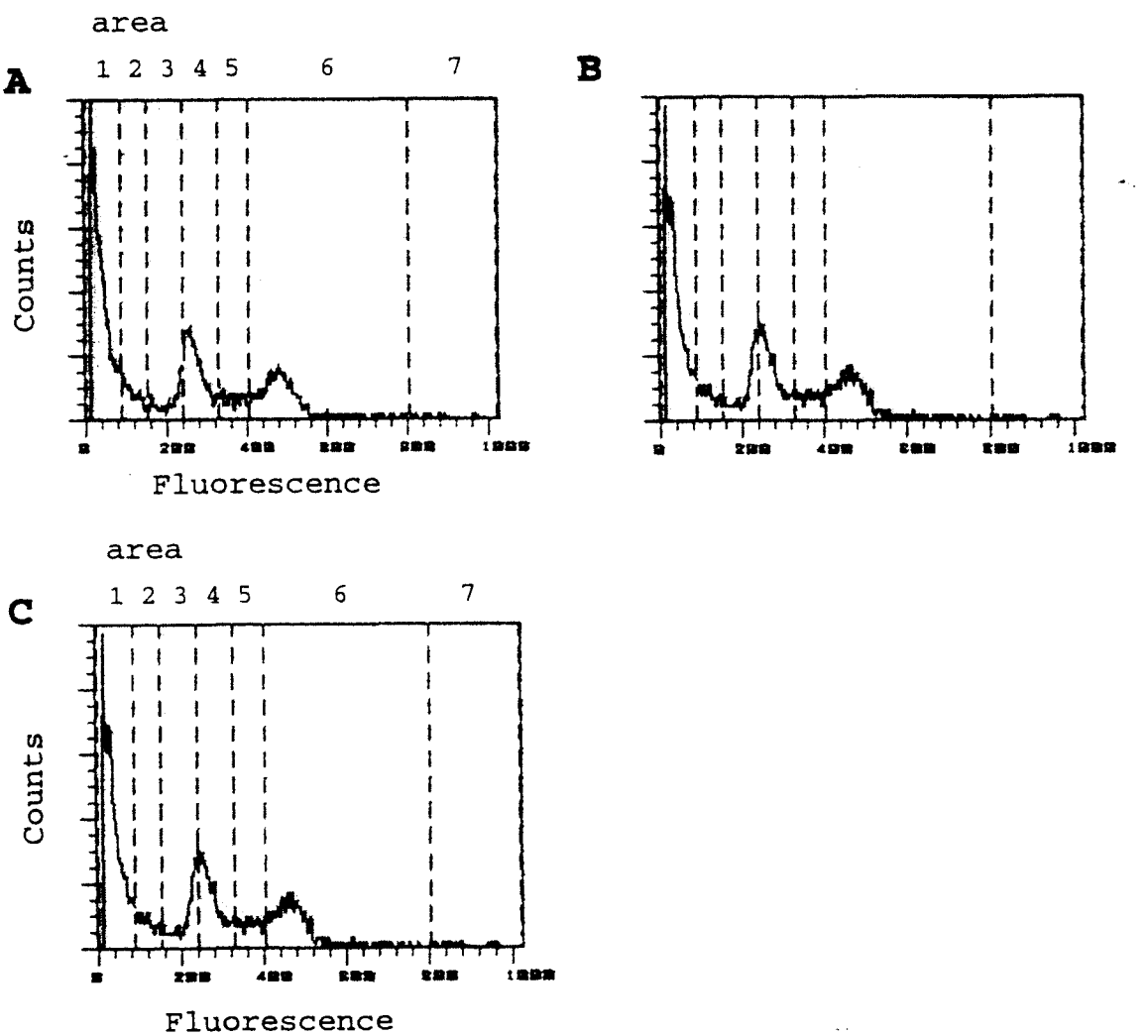

Figure 5. Flow histograms for various lengths of hypotonic treatment. A) 20 min, B) 40 min, C) 60 min. Chromosomes stained with 25 µg/ml PI.

Section	Total	Percent	%CV	Peak Height
1	1994	39.88	50.98	67
2	325	6.50	15.67	9
3	345	6.90	13.35	12
4	926	18.52	8.17	19
5	299	5.98	6.07	6
6	1038	20.76	14.88	11
7	33	0.66	6.42	1

A

Section	Total	Percent	%CV	Peak Height
1	1834	36.68	50.74	62
2	321	6.42	15.46	7
3	559	11.18	11.69	19
4	877	17.54	8.75	20
5	348	6.96	5.92	6
6	982	19.64	14.01	11
7	42	0.84	6.94	2

B

Section	Total	Percent	%CV	Peak Height
1	1851	37.02	51.36	65
2	257	5.14	15.33	6
3	429	8.58	11.93	16
4	916	18.32	8.46	17
5	314	6.28	5.74	6
6	1124	22.48	13.69	13
7	81	1.62	7.42	2

C

Table 4. Event distributions for flow histograms generated using different lengths of hypotonic treatment. A) 20 min, B) 40 min, C) 60 min. Chromosomes were stained with 25 µg/ml PI.

Vortexing

Flow histograms from the various times of vortexing are shown in figure 6 and the event distribution is shown in

table 5. As the amount of vortexing is increased, there is a corresponding increase in the number of events occurring in the first area of the flow histogram as well as a decrease in the height of the peak in the sixth area of the flow histogram.

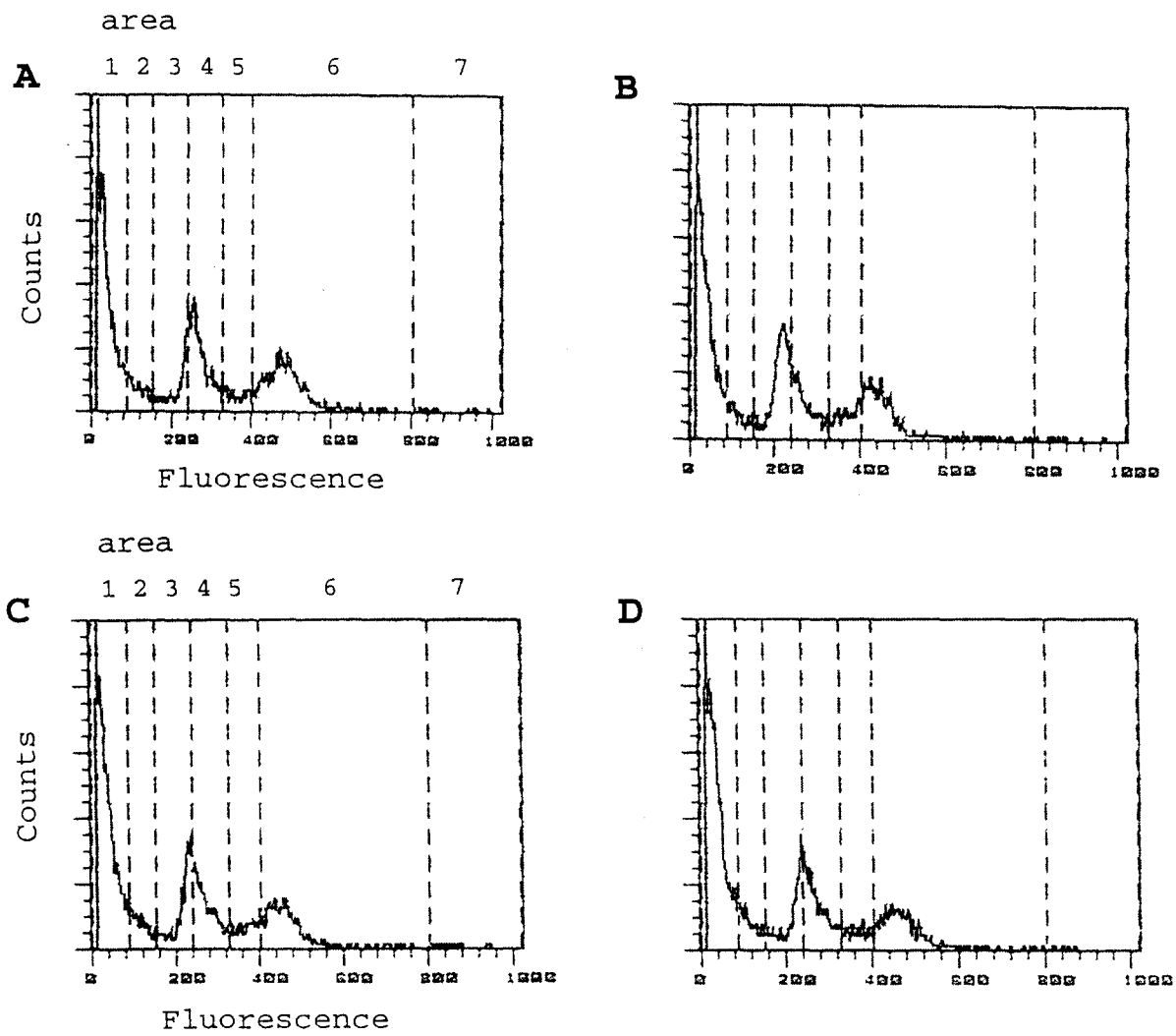

Figure 6. Flow histograms for various lengths of vortexing. A) 15 sec, B) 30 sec, C) 45 sec, D) 60 sec, E) 75 sec. Chromosomes were stained with 25 µg/ml PI.

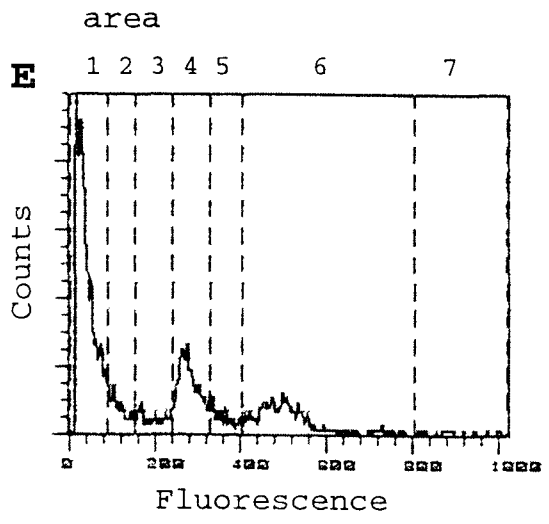

Figure 6 continued. Flow histograms for various lengths of vortexing. A) 15 sec, B) 30 sec, C) 45 sec, D) 60 sec, E) 75 sec. Chromosomes were stained with 25 µg/ml PI.

Section	Total	Percent	%CV	Peak Height
1	1792	35.84	51.37	63
2	294	5.88	16.12	8
3	415	8.30	12.40	18
4	971	19.42	8.29	23
5	257	5.14	6.24	5
6	1178	23.56	12.49	13
7	46	0.92	7.51	1

A

Section	Total	Percent	%CV	Peak Height
1	1846	36.92	50.56	64
2	287	5.74	16.28	7
3	913	18.26	9.52	22
4	587	11.74	9.11	14
5	371	7.42	5.96	8
6	929	18.58	16.66	12
7	35	0.70	5.70	1

B

Table 5. Event distributions for flow histograms generated using different times of vortexing. A) 15 sec, B) 30 sec, C)45 sec, D) 60 sec, E) 75 sec. Chromosomes were stained with 25 µg/ml PI.

Section	Total	Percent	%CV	Peak Height
1	1993	39.86	50.50	67
2	313	6.26	15.67	9
3	663	13.26	10.64	22
4	739	14.78	8.70	17
5	339	6.78	5.94	8
6	862	17.24	14.09	10
7	61	1.22	5.38	1

C

Section	Total	Percent	%CV	Peak Height
1	2112	42.24	49.79	70
2	343	6.86	15.81	9
3	547	10.94	11.77	23
4	785	15.70	8.85	21
5	278	5.56	5.92	6
6	850	17.00	14.31	9
7	29	0.58	2.68	1

D

Section	Total	Percent	%CV	Peak Height
1	2313	46.26	50.94	77
2	330	6.60	16.03	10
3	278	5.56	13.84	6
4	862	17.24	7.89	17
5	247	4.94	6.20	7
6	884	17.68	16.23	8
7	75	1.50	7.22	3

E

Table 5 continued. Event distributions for flow histograms generated using different times of vortexing. A) 15 sec, B) 30 sec, C)45 sec, D) 60 sec, E) 75 sec. Chromosomes were stained with 25 µg/ml PI.

Propidium Iodide Staining

The event distribution for the various PI concentrations is shown in table 6 (flow histograms are not available). As can be seen, differing concentrations of PI cause dramatic differences in the peak heights and peak distributions obtained. A concentration of 15 µg/ml gave a flow histogram closest to the optimal flow histogram (fig. 2).

Section	Total	Percent	%CV	Peak Height
1	2433	48.66	51.84	95
2	396	7.92	15.59	13
3	190	3.8	12.61	4
4	68	1.36	9.44	2
5	26	0.52	6.73	1
6	898	17.96	7.32	18
7	955	19.1	5.76	23

A

Section	Total	Percent	%CV	Peak Height
1	1961	39.22	48.41	54
2	261	5.22	16.15	7
3	147	2.94	12.71	3
4	70	1.4	8.92	2
5	115	2.3	6.28	6
6	2135	42.7	24.37	23
7	329	6.58	7.8	7

B

Section	Total	Percent	%CV	Peak Height
1	773	15.46	52.35	29
2	298	5.96	15.60	24
3	2118	42.36	11.85	66
4	768	15.36	8.98	13
5	747	14.94	4.37	22
6	274	5.48	16.83	2
7	0	0.00	0.00	0

C

Section	Total	Percent	%CV	Peak Height
1	4867	97.34	47.69	289
2	101	2.02	16.37	10
3	21	0.42	4.04	1
4	0	0.00	0.00	0
5	0	0.00	0.00	0
6	0	0.00	0.00	0
7	0	0.00	0.00	0

D

Table 6. Event distibution for differing concentrations of propidium iodide. A) 50 µg/ml, B) 15 µg/ml, C) 5 µg/ml, D) 0.5 µg/ml

Hybridized Chromosomes

Hybridized chromosomes produced a flow histogram with greatly reduced peaks as well as missing peaks (fig. 8). Vortexing the sample after each centrifugation step of the hybridization procedure resulted in a histogram with slightly larger peaks (table 7).

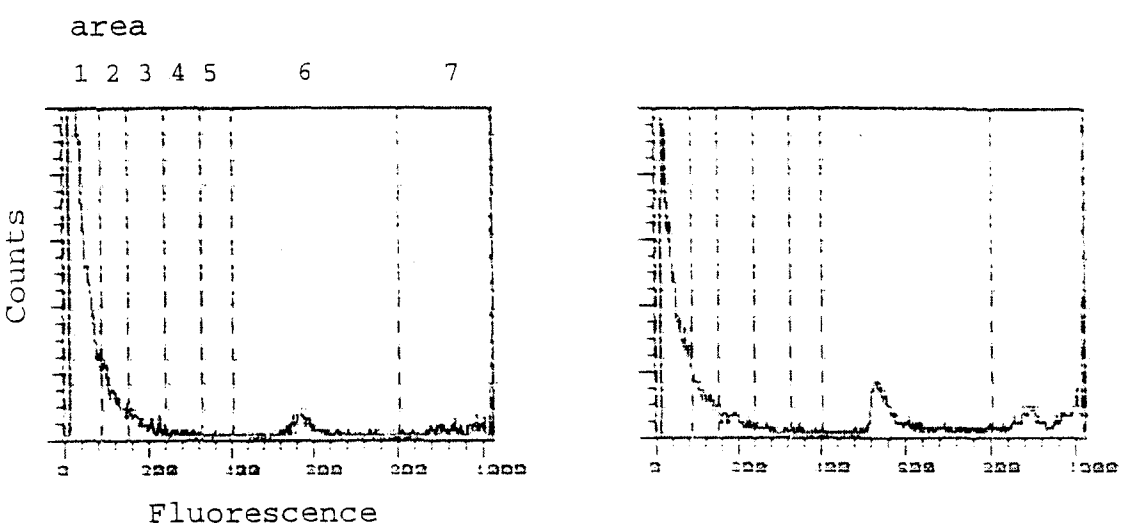

Figure 8. Flow histograms for hybridized chromosomes with and without vortexing (left histogram = no vortexing). Chromosomes were stained with 25 µg/ml PI.

Section	Total	Percent	%CV	Peak Height
1	3261	61.14	49.77	98
2	569	10.67	15.94	15
3	277	5.19	13.29	7
4	107	2.01	8.86	2
5	70	1.31	5.64	2
6	549	10.29	14.69	6
7	493	9.24	6.61	42

A

Section	Total	Percent	%CV	Peak Height
1	2256	42.38	50.47	62
2	510	9.58	15.77	12
3	307	5.77	12.82	5
4	147	2.76	9.37	3
5	83	1.56	6.19	3
6	1014	19.05	15.41	11
7	1016	19.09	6.99	106

B

Table 7. Event distributions for flow histograms generated from hybridized chromosomes with and without vortexing after centrifugations. A) without vortexing, B) with vortexing.

DISCUSSION

CHROMOSOME STABILITY

Chromosome stability has been an important factor in the preparation of isolated chromosomes for flow cytometry. Each of the three main buffers used for chromosome isolation allows preservation of chromosome morphology to varying

degrees (Bartholdi et al, 1987). The polyamine buffer first used by Sillar and Young (1981) seems to produce chromosomes that maintain good morphology for the longest storage times, up to 18 months. Chromosomes isolated with the hexylene glycol and magnesium sulfate buffers require storage in a freezer or must be used within one week, respectively. Freezing of the isolated chromosomes was thought to prevent nuclease activity present in the cell extract. This was supported by the fact that addition of nuclease inhibitors to the isolation buffers made chromosomes better able to survive long term storage. However, when chromosomes isolated in the presence of nuclease inhibitors are stored for long periods at 4° C and then exposed to denaturing temperatures, their structure is lost or degraded, suggesting that more than nuclease activity is involved in chromosome stability.

The polyamines spermine and spermidine present in the polyamine isolation buffer bind to the negatively charged phosphate groups on the DNA backbone and provide stability to the chromosomes. Experiments have shown two possible models for this polyamine binding; one in which the polyamine molecule binds to phosphate groups on one strand of DNA (intrastrand binding) and one in which the polyamine molecule binds to phosphate groups on both strands of DNA

(interstrand binding). Neither method alone completely explains the mechanisms of polyamine and DNA interaction and both models are thought to be occurring in unison (Glaser and Gabbay, 1968). Experiments have also shown high levels of polyamines will cause precipitation of the polyamine-DNA complexes (Razin and Rozansky, 1959) suggesting polyamine binding to multiple DNA molecules.

During interstrand binding, spermine, the larger of the two polyamines, covers a total distance of approximately 2.8 nM (Bachrach, 1973). When DNA is bound to histones in the nucleosome, the distance between each coil of DNA is 2.7 nM. It is probable that each of the terminal amine groups of the spermine molecule binds to a phosphate group on different, but juxtaposed, DNA helices in the nucleosome. With spermidine bound in this manner, the chromosome would maintain its characteristic shape even if the histones were completely removed. Further support for this line of reasoning comes from the fact that chromosomes in the presence of polyamines are resistant to degradation with trypsin or elongation via histone removal by 0.70 M KCl (unpublished data).

While spermine and spermidine preserve the chromosome structure for routine flow cytometry, the polyamine binding is electrostatic and is disrupted during the harsh denaturation conditions of FISH in suspension, thereby

removing any support they previously provided. Because the fixation and hybridization steps remove the excess polyamines, their concentration after denaturation is low and prevents them from reassociating with the DNA at any appreciable level. The overall result of the hybridization procedure then is the removal of the polyamines and the structural support they provided.

For some cell lines, such as HSF-7, the chromosome structure is completely lost during the denaturation step of FISH in suspension. For other cell lines, such as human-hamster hybrid cell lines, the chromosomes are able to survive the denaturation step with little or no loss of chromosome morphology. A possible explanation for this is different levels of protease activity between human cell lines and human-hamster hybrid cell lines. To test this, I included a protease inhibitor cocktail in the chromosome isolation buffer. Inclusion of this protease inhibitor did indeed allow the chromosomes derived from HSF-7 cells to better survive the denaturation step. However, after storage at 4° C and denaturation, the chromosome morphology became progressively worse until eventually no good chromosome morphology remained. I therefore decided to fix the chromosomes immediately after isolation using a standard methanol:acetic acid fix (3:1) to eliminate any remaining

protease or nuclease activity. This prevented degradation of the chromosomes and allowed them to be hybridized even after four weeks storage at 4° C.

FLOW CYTOMETRIC ANALYSIS

Chromosomal and cellular debris form a continuum in the first area of the flow histogram. Therefore, an increase in the number of events occurring in the first area, relative to the total number of events, is taken to represent an increase in the total debris level.

As the length of time the cells are exposed to Colcemid is increased, there is an increase in the levels of debris observed, presumably from micronuclei formation and Colcemid toxicity (Bartholdi et al, 1987). For the GM 130B cell line, six hours of Colcemid treatment results in the best flow histogram.

The length of time the cells remained in the hypotonic treatment did not have an appreciable effect on the flow histogram. Therefore, the standard cell culturing time of 20 min was used.

The amount of vortexing used during the chromosome isolation procedure is very important for obtaining good

flow histograms and for chromosome sorting. Too little vortexing results in clumps of chromosomes, and a corresponding decrease in the number of analyzable monodispersed chromosomes, while too much vortexing results in fragmentation of the monodispersed chromosomes and, again, a reduction in the number of analyzable monodispersed chromosomes, as well as an increase in the level of chromosomal debris. For the GM 130B derived chromosomes, 15 sec of vortexing gives the lowest amount of debris and the highest number of analyzable chromosomes relative to the total number of events (i.e. the highest chromosome peaks per run).

The amount of propidium iodide used for chromosome staining has a dramatic effect on the flow histogram. Even small variations in the concentration used can make the difference between excellent and unusable results. For the GM 130B cell line, 15 µg/ml PI gave the best flow histogram.

For hybridized chromosomes, the peaks of the flow histogram are greatly reduced. This may be due to clumping of the chromosomes caused during the centrifugation steps of the hybridization procedure. Vortexing the sample after each centrifugation resulted in larger peaks in the histogram. However, to obtain a good analyzable histogram, it will be necessary to develop protocols to reduce or

remove the clumping to allow for the analysis of monodispersed chromosomes.

Conclusions

I have shown here that fluorescence in situ hybridization to isolated human chromosomes in suspension is not only possible, but, following our protocol, is a robust, straight forward and reproducible procedure. The optimum conditions for the GM 130B cell line for chromosome isolation were found to be: 6 hrs Colcemid treatment, 20 min hypotonic treatment, 15 sec vortexing and 15 µg/ml propidium iodide staining. I have also demostrated the feasability of flow cytometric analysis of the hybridized chromosomes. The hybridized chromosomes produce a flow histogram showing the same relative peaks and event distributions as flow histograms from unhybridized chromosomes. However, there is a higher background level which may be due to clumping of chromosomes and chromosomal debris. To produce an optimal flow histogram from hybridized chromosomes, it will be necessary to develop protocols that reduce the clumping of the chromosomes that occurs during the hybridization procedure.

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