Development and Application of Comparative Genomic Hybridisation (CGH)

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To: Jila

"With them the Seed of Wisdom did I sow,

And with my own hand labour'd it to grow:

And this was all the Harvest that I reap'd---

I came like Water, and like Wind I go."

(Rubayyat of Omar Khayyam)

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DECLARATION

I certify that this thesis does not contain material previously published or written by any other person except where referred to in the text and that the results of this study have not been submitted for any other degree or diploma. Parts of the results of this study are published in journals, or presented in conferences or meetings as indicated in the next page.

S.R. Ghaffari

LIST OF PUBLICATIONS

Ghaffari SR, Boyd E, Tolmie JL, Crow YJ, Trainer AH, Connor JM. A new strategy for cryptic telomeric translocation screening in patients with idiopathic mental retardation. *J Med Genet* 1998,35:225-233

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Ghaffari SR. CGH for constitutional chromosome abnormalities and prospects for CGH to DNA microarray targets. *J Med Genet* 1998:35 (SP03) (British Human Genetics Conference September 1998)

OTHER PRESENTATIONS

Cytogenetics, past, present and future. Cytogenetic Symposium, Glasgow 23rd May 1997

Comparative genomic hybridisation in clinical genetics. University of Glasgow seminar in Medical Genetics, 11th February 1998

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LIST OF ABBREVIATIONS

A	Adenine
ASO	Allele specific oligonucleotide hybrids
bp	Base pair
BAC	Bacterial artificial chromosome
C	Cytosine
°C	Degree Celsius
CCD	Charge coupled device
CGH	Comparative genomic hybridisation
CISS	Chromosomal in situ suppression
cm	Centimetre
DAPI	4'-6-diamidino-2-phenylindole
del	Deletion
ddATP	Dideoxyadenisine triphosphate
ddCTP	Dideoxycytosine triphosphate
ddGTP	Dideoxyguanosine triphosphate
ddTTP	Dideoxythymidine triphosphate
ddNTPs	Dideoxyribonucleotide triphosphate
DMS	Double minutes
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
dNTP	2'-deoxyribonucleoside triphosphate
DOP-PCR	Degenerate oligonucletide primers PCR
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
FISH	Fluorescence *in situ hybridisation*
FITC	Fluorescein isothiocyanate
G	Guanine
g	gram
hMSH2	Human mut S homologous 2
ISH	*in situ* hybridisation
kb	Kilobase
kd	Kilo dalton
M	Molar
Mb	Megabase
MES	2-(N-morpholino) ethanesulfonic acid
μg	Microgram
μl	Microlitre
mg	Miligram
ml	Mililitre
μM	Micro molar
mM	Milimolar
mm	Milimetre
ng	Nanogram
OD	Optical density
p	Short arm of chromosome
PAC	P1-derived artificial chromosome
PHA	Phytohaemagglutinin
PCR	Polymerase chain reaction
pmol	Picomole
q	Long arm of chromosome
r	Ring chromosome
RBC	Red blood cells
RNA	Ribonucleic acid
ROI	Region of interest
rpm.	Revolutions per minute
SCLC	small cell lung carcinoma
SD	Standard deviation

SDS	Sodium dodecyl sulphate
SSC	Saline sodium citrate
T	Thymine
t	Translocation
TBE	Tris-acetate ethylenediaminetetra acetic acid
TE	Tris- ethylenediaminetetra acetic acid
ter	Terminal
TR	TexasRed
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet
V	Volume
W	Watt
WCP	Whole chromosome paint
X	Human X chromosome
Y	Human Y chromosome
YAC	Yeast artificial chromosome

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SUMMARY

The aims of this study were first, to optimise the comparative genomic hybridisation (CGH) technique and test its reliability as a diagnostic tool in a clinical genetics laboratory, second, to investigate whether any of the current limitations could be overcome, and third, to improve the resolution of the technique beyond the current limits by targeting DNA molecules instead of metaphase chromosomes.

During the course of this study, a modified approach to CGH was developed and tested on samples with known and unknown abnormalities in various fields of clinical cytogenetics. The technique was used to detect unbalanced abnormalities in patients with common constitutional abnormalities, marker chromosomes, mosaicism, haematological malignancies and solid tumours. In all fields CGH detected some abnormalities which had remained undetected using conventional cytogenetics or molecular cytogenetic techniques.

During the optimisation 94 samples were used. Samples with known karyotypes were the subject of CGH using different conditions of slide preparation, slide treatment, DNA isolation (from different samples including fixed cells) DNA labelling, hybridisation, detection and washing approaches. Some modifications in the standard procedures were adopted and then the modified approach was applied to 56 cases with different abnormalities.

Overnight CGH was successfully developed and applied during this study. Nineteen experiments using 10 test DNAs all correctly detected the abnormalities in the cases. 10 samples were the subject of CGH using samples amplified by degenerate oligonucletides primers PCR (DOP-PCR). CGH reliably detected unbalanced abnormalities in the affected cases using as little as 1 ng of DNA.

CGH was applied on 14 samples from patients with haematological malignancies. In 2 cases CGH did not detect the abnormality found by conventional cytogenetics because of the balanced nature of the rearrangements. Both CGH and conventional cytogenetics found no abnormality in 5 samples. In 3 abnormal cases CGH and G-banding results

were compatible. In 3 patients, CGH detected abnormalities missed by routine cytogenetic analysis. These abnormalities were detected in mosaicism of as low as 30% of abnormal cells.

CGH detected new abnormalities that were not detected by conventional cytogenetics in a case of follicular lymphoma. A new site of DNA amplification at chromosome 3p26 was also detected. A new method for defining the copy number differences in cases with ploidy changes was suggested and was successfully applied in a case with hypotriploidy.

13 mentally handicapped patients with normal karyotypes without any clinical clue to a specific syndrome were the subjects of CGH at the next stage of this study. Using a new approach CGH detected telomeric gains and losses in 5 patients from 3 families. Those abnormalities were then confirmed using targeted FISH. Balanced carriers of the translocations in three generations were then detected using targeted FISH.

Comparative genomic hybridisation revealed the origin of a mosaic supernumerary ring chromosome 19 in a 72 year-old woman with mental retardation. That abnormality could not be identified by conventional banding methods over many years and, more recently, fluorescence in situ hybridisation (FISH) studies which systematically employed different chromosome probes. In a female with mental retardation and a putative iXp, CGH ruled out that diagnosis by detecting the presence of Xq in the DNA material from the patient. CGH then reliably defined the abnormality as an Xp;Xq translocation.

To improve the resolution of the CGH technique combed DNA fibres instead of metaphase chromosomes were used as the target DNA for the hybridisation of specific probes. CGH was then successfully performed on high-density DNA fibres of 35 kb nucleotides. At the final stage of this study CGH was performed on spots of single stranded DNA arrays of ~300-500 bp corresponding to chromosomes X, Y, and chromosome 2 on glass slides. Preliminary results showed that this approach could differentiate the copy number differences using a statistical analysis.

This study shows that CGH is a powerful adjunct to the current techniques in clinical cytogenetics for detection of unbalanced abnormalities in the various fields of clinical genetics. This study also presents several modifications in CGH technique and image analysis which improve the resolution and the reliability of the technique and make it faster and less complex. It also provides an opportunity for future studies for CGH on DNA microarray targets that would be a very important step towards the complete automation of the cytogenetics.

1 :Introduction

1.1 Background

In 1866, Mendel showed that many characteristics were passed on as individual, separate factors, which came to be called genes. These are transmitted from one generation to the next in a predictable, regular manner. In 1903, the cytologists Boveri and Sutton independently noticed intriguing parallels between the way in which the hypothetical Mendelian genetic factors behaved, and the distribution of real, visible structures inside cells, the chromosomes.

Cytogenetics is the study of chromosomes by microscopy. The exact number of human chromosomes in somatic cells was determined as 46 by Tjio and Levan in 1956. This finding was quickly followed by the discovery that abnormal chromosome numbers were present in patients with Down's syndrome (Lejeune et al 1959). This, together with the introduction of the PHA-stimulated lymphocyte culture technique (Moorehead et al 1960) marked the beginning of clinical cytogenetics, although since chromosomes were stained with feulgen or giemsa, only gross abnormalities could be detected.

In 1970, Pardue and Gall developed the in situ hybridisation (ISH) technique. They showed that labelled DNA probes could be annealed to complementary DNA sequences in cytologic preparations of chromosomes made by standard techniques. Pardue and Gall also noted that when the denatured chromosomes were stained by Giemsa, the paracentric regions were preferentially stained (C bands). Various modifications of the denaturing and staining process by other scientists following Pardue and Gall yielded chromosomes that showed patterns of differential staining along their length, which appeared specific for each chromosome. These experiments led to the establishment of standard chromosome banding patterns (Caspersson et al 1970; Seabright 1971), which have become the criteria for chromosome identification and classification in human cytogenetics.

Chromosome banding techniques have facilitated the identification of specific human chromosomes and presently provide the major basis upon which chromosomal aberrations are diagnosed. The interpretation of chromosome banding patterns requires skilled personnel and is often technically difficult, especially with respect to detecting minor structural changes and when analysing complex karyotypes, such as those of highly aneuploid tumour cells. An additional complexity is that readable metaphase chromosome spreads are sometimes very difficult or impossible to prepare from certain cell types or tissues.

While the initial *in situ methodology* was a powerful tool for gene mapping, the high background caused by the isotopic probes reacting with the sensitive photographic emulsion imposed significant limitations in terms of time and precise localisation of the target sequence. The search for non-isotopic alternatives for labelling probes for ISH led first to the introduction of biotin (Langer et al 1981) and then other haptens including acetylaminofluorene (Landegent et al 1984), mercury (Bauman et al, 1983), digoxigenin (Heiles et al 1988; Seibl et al 1990), and sulphonate (Lichter et al 1988). These methods require indirect detection using specific antibodies. However, in recently introduced direct labelling methods the fluorochromes such as FITC, Cy3 and TexasRed are coupled directly to the nucleotides that are used in the labelling of the DNA probes.

In 1980s, molecular biology coupled with cytogenetics, resulted in the emergence of a new discipline, molecular cytogenetics (Landegren et al 1988). The development of FISH and the generation of numerous chromosome-specific DNA probes have provided new possibilities for complementing chromosome banding techniques. Interphase cytogenetics has allowed the assessment of numerical and structural chromosome aberrations. Multiple colour FISH has further enhanced the capacity for identifying chromosome aberrations with speed and accuracy (Nederlof et al 1990; Reid et al 1992). However, in order to select DNA probes useful for the detailed analysis of a clinical sample, previous knowledge of the types of expected aberrations is required.

Molecular genetics has provided additional powerful tools for use in the search for genetic imbalances in genomic DNA. This approach requires large numbers of polymorphic DNA markers informative for the patient in question. Although amplifications of specific DNA sequences can easily be detected, e.g., in Southern blots from genomic DNA, it might be difficult to choose the appropriate probes. It is even more difficult to distinguish between two and three copies of a DNA segment. The global analysis of genomic DNA from tumour samples for chromosomal gain and losses by presently available methods of molecular genetics therefore remain too laborious to be implemented in routine diagnostic schemes.

Comparative genomic hybridisation (CGH) is a new molecular cytogenetics technique, which was first described by Kallioniemi et al (1992). CGH is a genome-wide screening method in which differentially labelled test and control DNAs are co-hybridised to a normal target metaphase to assay the copy number differences and provides a new global approach for searching clinical and tumour specimens for genetic imbalances in a single hybridisation.

1.2 The cell, the chromosome, and the DNA

1.2.1 The *cell*

Visible with the light microscope within each cell of the body are the *cytoplasm* and a darkly staining body, the *nucleus.* The cytoplasm has within it a complex arrangement, the *endoplasmic reticulum,* small bodies concerned with cell respiration, the *mitochondria,* and the *ribosomes,* involved with protein synthesis. The nucleus is surrounded by a membrane which separates it from the cytoplasm and within the nucleus are the chromosomes which bear the genes.

1.2.2 Structure of DNA

The basic components of DNA are 2-deoxyribose, and four heterocyclic bases: two purines (adenine and guanine), and two pyrimidines (thymine and cytosine). The sugar residues are linked by phosphodiester bonds between

the 5' position of one deoxyribose and the 3' position of the next, while one of the four bases is attached to the 1' position of each deoxyribose. The two strands are linked by means of hydrogen bonds between the purine bases and the pyrimidines (Figure 1. 1).

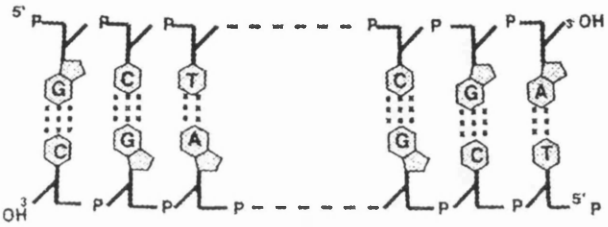


Figure 1. 1: Specific pairing of the two strands of the DNA

1.2.3 Sequence architecture of the human genome

The haploid human genome consists of 3000 million base pairs (3000 Mb) of DNA. It consists of different sequence classes: single sequence or low copy DNA, intermediate repeated DNA sequences consisting of families of DNA sequences repeated between 10^2 and 10^5 times per genome and, highly repetitive or satellite DNA repeated 10^6 times per genome.

There are two different classes of intermediate repeat sequences called SINEs and LINEs. SINEs and LINEs are mobile genetic elements that undergo selection to increase in number but do not contribute to the phenotype. The most common LINE is called L1. The most common SINE is a family of sequences called *Alu*.

Most satellite DNA is found in very long tandem arrays in heterochromatic regions around the centromere. An important example of a satellite DNA sequence is α-satellite DNA which has an essential function in centromeres of chromosomes. Minisatellites consist of tandem repeats of units 10-100 bp in size. The number of repeats and the sequence of the repeat units are both polymorphic.

1.2.4 Human chromosomes

The 6000 Mb of DNA that constitutes the human genome is divided between 23 pairs of chromosomes; 22 of these chromosomes are present in both

males and females and are defined as autosomes. The remaining pair is different in males and females and is known as the sex chromosomes.

Chromosome structure

A single DNA molecule runs the length of each chromosome. This molecule is about 250 Mb in size in the largest human chromosome (chromosome 1). Physically the DNA in chromosome is packaged at mitotic metaphase into a chromosome in a compaction ratio of about 10000 to one. The basic unit is called a nucleosome, a cylinder about 8x11 nm in size, in which 140 bp of DNA is wound around a histone core containing two molecules each of the four core histones: H2A, H2B, H3, and H4. Each nucleosome is linked to the next by a 60 bp long piece of naked DNA. There are about 74 nucleosomes per average gene of 15-kb length, up to 6.5 $x10^5$ nucleosomes per average chromosomes, and up to $30x10^6$ nucleosomes per nucleus. DNA wound up in nucleosomes is coiled again to form a fibre 25 to 30 nm in diameter. The 30 nm fibre is stabilised by a fifth histone called histone H1. Finally, at mitosis the 30 nm fibre winds around a scaffold of protein to form a fibre 700 nm in diameter. Various non-histone proteins are also associated with the DNA. The whole DNA-RNA-protein complex is known as chromatin. During interphase, chromosomes are not so tightly condensed as in metaphase but the DNA must still be highly packaged.

1.3 Chromosomes in clinical medicine

1.3.1 Chromosome aberrations

Genetic mutations involve deletion, duplication, or rearrangement of DNA. Those changes that are large enough to be detectable under the light microscope are classified as chromosome aberrations. The overall rate of chromosome aberrations at birth is about 0.6% (0.35% numerical, 0.23% structural, and 0.02% both).

Numerical aberrations

Numerical aberrations involve the gain or loss of one or more chromosomes (aneuploidy), or the addition of one or more complete haploid complements (polyploidy).

The failure of paired chromosomes or sister chromatids to separate at anaphase (non-disjunction) and anaphase lag resulting in chromosome loss are the usual causes of aneuploidy. Gain of one or two homologous chromosomes is referred to as trisomy and tetrasomy respectively. Loss of a single chromosome results in monosomy. The most common numerical abnormalities are shown in Table 1. 1.

Table 1. 1: Incidence on common numerical chromosomal abnormalities

Trisomy 21	1/800
47,XXX	1/960
47,XXY	1/1080
47,XYY	1/1080
Trisomy 18	1/8140
45,X	1/9600
Trisomy 13	1/19000

Structural aberrations

Structural chromosome rearrangements result from chromosome breakage with subsequent reunion in a different configuration and can be balanced or unbalanced. In balanced rearrangements, the chromosome complement is complete with no loss or gain of genetic material and therefore they are generally harmless unless one of the breakpoints damages an important functional gene. Balanced carriers may have balanced or unbalanced offspring. In individuals with unbalanced chromosomal abnormalities the chromosomal complement contains an incorrect amount of chromosome material causing severe clinical effects. The main types of balanced and

unbalanced structural abnormalities are shown diagrammatically in Figure 1. 2 and Figure 1. 3.

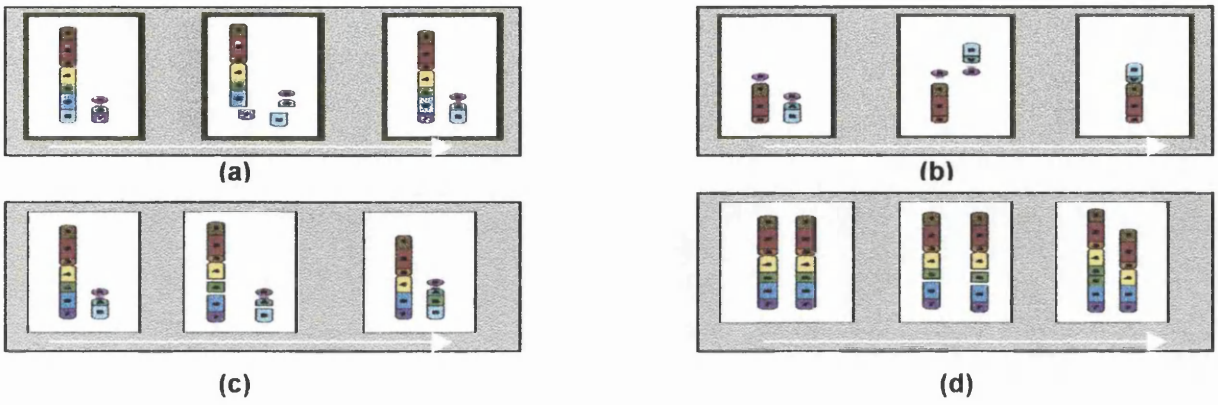


Figure 1. 2: Structural abnormalities: (a) reciprocal translocation (b) robertsonian translocation (c) insertional translocation (d) deletion//duplication

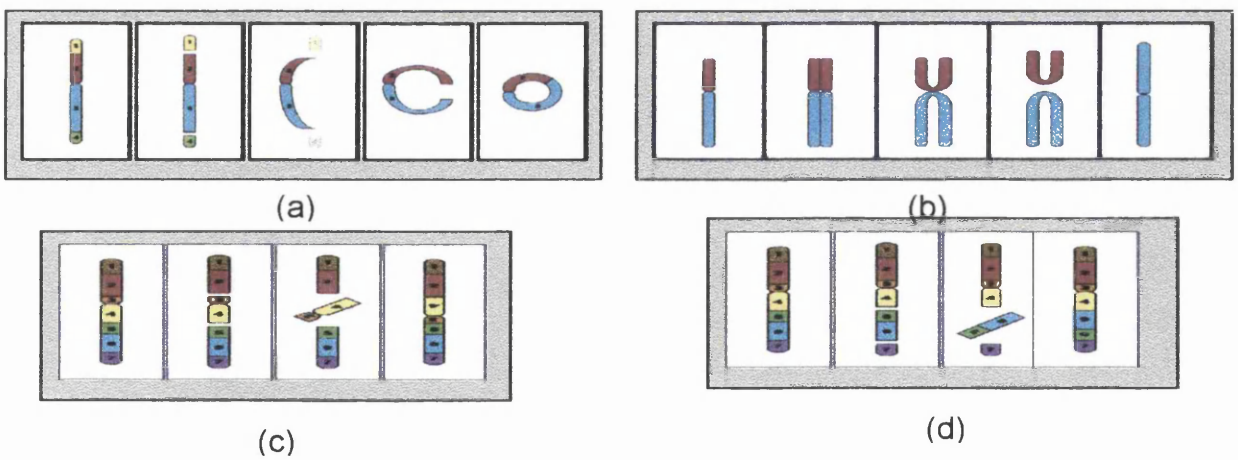


Figure 1. 3: Structural abnormalities: (a) ring chromosome (b) isochromosome of long arm (c) pericentric inversion (d) paracentric inversion

1.3.2 Cryptic chromosomal aberrations

By 1995 there were several case reports of subtle or "cryptic" chromosomal aberrations mainly involving telomeric ends of chromosomes. These events were not detected by conventional cytogenetic methods but were detected by molecular cytogenetic methods (Lamb et al 1989; Ledbetter 1992; Wilkie 1993). A cryptic chromosomal aberration is therefore defined as one that is sufficiently subtle enough that it would not be reliably detected, in the majority of diagnostic laboratories, by G-band analysis at the 400-500 band stage of resolution. These findings showed the necessity of improving resolution of the conventional techniques or molecular cytogenetic methods or using molecular techniques.

1.3.3 Chromosomes in malignant diseases

Chromosome aberrations are common in malignant cells. Certain chromosomes seem to be more commonly involved in particular malignancies. Over 70 human neoplasms are known to have one of the more than 40 specific recurrent chromosomal abnormalities that can be either translocations, deletions, duplications, inversions, or trisomies. Translocations are seen in leukaemia and non-Hodgkin's lymphoma, whereas deletions are found most frequently in myelodysplasia and solid tumours. At present, the most important practical benefit of cytogenetic analysis in haematological malignancies is in helping to determine prognosis, although the identification of specific translocations can also aid in the diagnosis. More accurate determination of the prognosis for remission and long-term disease-free survival will, in turn, probably have a major impact on therapeutic recommendations.

Cytogenetics and molecular biology have each successfully identified non-random alterations in the genome that are associated with malignant disorders. Growth of solid tumours in culture for cytogenetic analysis is poor and is compounded by low mitotic indices. Often the specimens are contaminated with bacteria and other microbial agents and may contain large regions of necrotic tissue. The major clone that does grow may not reflect its true representation in the tumour in vivo. In addition, karyotyping is often

hampered by the low number of high quality metaphase spreads, low percentage of the abnormal cells and/or the complex nature of chromosomal changes.

1.4 Methods of chromosome analysis

1.4.1 Cell Culture

Most cytogenetic studies involve the examination of a dividing cell population by blocking cell division at metaphase with an inhibitor of spindle formation. Normal circulating blood cells do not divide under routine culture conditions. Several mitogens such as phytohaemagglutinin (PHA), pokeweed mitogen and concanavalin A can stimulate the proliferation of blood cells. The peak mitotic activity is reached after 60-70 hours of culture and is the optimum harvesting point for chromosome studies. However, 48 to 90-hour cultures may be used.

1.4.2 Harvesting and Slide Preparation

During the harvest procedure a mitotic inhibitor accumulates metaphases by preventing the formation of the spindle apparatus. Then, addition of the hypotonic solution causes water to rush into the cell and the cytoplasmic membrane stretches as the cell swells. Cells are preserved in this situation by fixative. When the fixed cell suspension is dropped onto a slide, the fixative immediately becomes a thin layer and does not support the cells any more. Evaporation of the fixative causes the cell to flatten and forces the chromosomes to spread out.

1.4.3 Banding techniques

Several different staining methods can be utilised to identify individual chromosomes.

Solid Staining

The chromosomes are exposed to a stain that has an affinity for DNA (like Giemsa), yielding a uniformly dark appearance to the chromatids. Solid staining has applications in the investigation of fragile sites, in the study of chromosome breakage syndromes, and for scoring radiation damage. It is also applied in the determination of marker chromosomes, the extent of satellite polymorphism, and in the measurement of individual chromosomes.

G (Giemsa) banding

This is the method most commonly used. The chromosomes are treated with trypsin which denatures their protein content and then with Giemsa stain which gives each chromosome a characteristic and reproducible pattern of light and dark bands. The dark bands contain mainly AT-rich DNA, and the light hands are CG-rich. G banding generally provides high quality chromosome analysis with approximately 400 bands per haploid genome. Each of these bands corresponds to approximately 8 Mb of DNA.

High resolution banding

High resolution banding of the chromosomes up to 800 bands per haploid genome involves first inhibiting cell division with the folic acid antagonist, methotrexate followed by adding folic acid in the medium which releases the cells into mitosis. Colchicine is then added at a specific time interval, when a higher proportion of cells will be in prometaphase and the chromosomes will not be fully contracted, giving a more detailed banding pattern. This method can detect quite subtle changes and has led to the definition of several microdeletion syndromes.

Other banding techniques

Some other banding techniques with specific applications are shown in Table 1. 2

Table 1. 2: List of some other banding techniques

Technique	Banding pattern	Main applications
Q-banding	Similar to Giemsa	identification of the Y heterochromatic region, study of the satellite regions of the acrocentric chromosomes
DAPI (4'-6-diamidino-2-phenylindole) banding	similar to Q-banding	highlights the centromeres of chromosomes 1, 9, and I 6
C-Banding	heterochromatic regions are preferentially stained	Study of centromeres and other heterochromatic regions
R (Reverse) banding	reverse of those obtained using conventional G banding.	useful for the definition of telomeric regions

1.4.4 Fluorescence in situ hybridisation (FISH)

Denaturation and hybridisation principles

FISH is the most commonly used molecular cytogenetic technique. FISH is based on the denaturation and hybridisation principles (Figure 1. 4). Since the two strands of DNA are only linked by non-covalent forces, they can easily be separated in the laboratory, for example by increased temperature or high pH (denaturation).

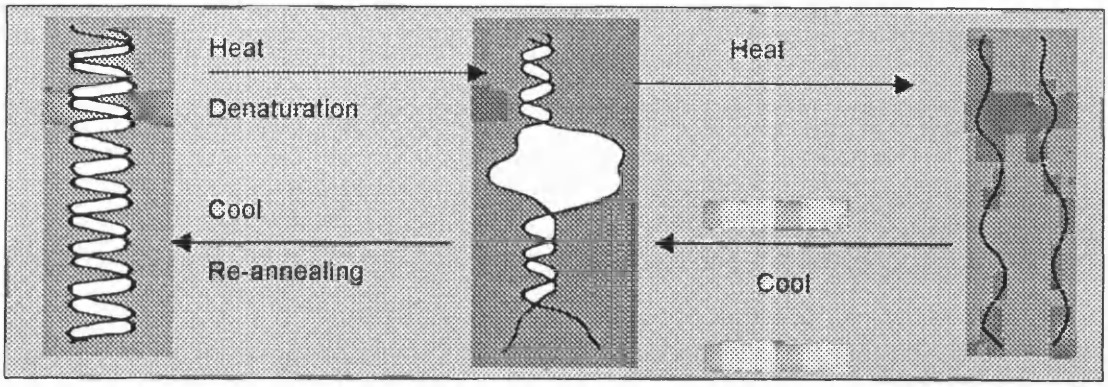


Figure 1. 4: Denaturation and hybridisation of DNA: As a double helix is heated those regions with less sequence homology between the strands, or those with a high proportion of relatively weak AT base pairs come apart; eventually, the whole helix separates. If an appropriately labelled DNA sequence (probe) is denatured and added to denatured nuclei or chromosomes during the process of re-annealing, some of that labelled DNA will hybridise to its complementary sequence in the chromosomal DNA. Detection of the labelled DNA will identify the site of hybridisation and thus the region of chromosomal DNA complementary to the DNA sequence in the labelled probe.

Reducing the temperature or the pH will allow hydrogen bonds between complementary DNA sequences to reform; this is referred to as re-annealing. Hybridisation between single-stranded DNA molecules forms the basis of the use of DNA probes to detect specific sequences in samples of DNA. The specificity of the reaction can be adjusted by altering the temperature and/or the ionic strength. Higher temperature or lower ionic strength gives greater stringency of hybridisation, i.e, the probe has to be more like the sequence to be detected. Outline of the FISH technique is depicted in Figure 1. 5.

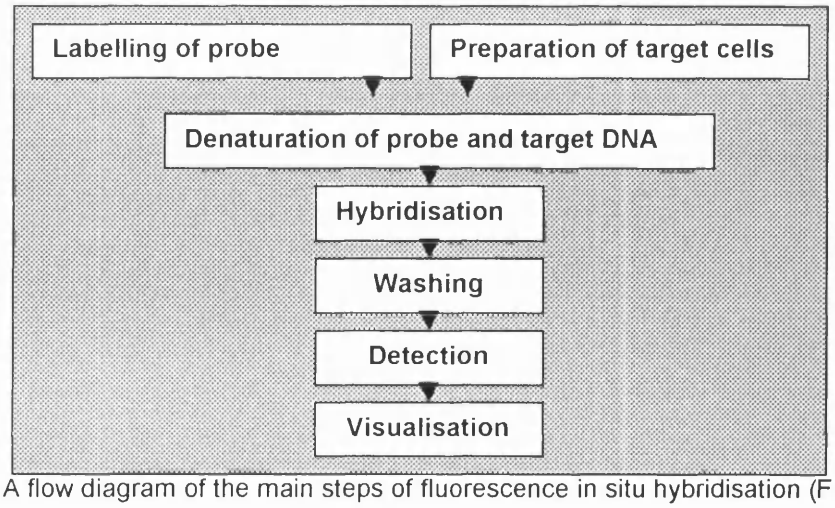


Figure 1. 5: A flow diagram of the main steps of fluorescence in situ hybridisation (FISH).

Probe labelling

Nick-translation is the most commonly used technique for probe labelling. In this method, DNase randomly nicks the double-stranded DNA probe, and then DNA polymerase is used to fill in the nicked strand with newly synthesised DNA containing the hapten-nucleotide. Similarly, in the PCR method, the labelled nucleotide is added to the reaction mixture. Probe-specific primers can be used for probe sequences of about 1 kilobase (kb), but larger sequences, for example, cosmid clones, are best labelled using degenerate oligonucleotide primed-PCR (DOP-PCR, Telenius et al 1992).

Direct and indirect detection

Biotin and digoxigenin have proved to be the most useful haptens, and a variety of methods have been developed to detect both these labels in cytologic preparations using fluorescence or enzyme-linked reactions. Probes labelled with either biotin or digoxigenin as reporter molecules could be visualised by indirect detection of hybridised probe via avidin or antidigoxigenin. The biotin has a strong affinity for avidin and streptavidin. When the latter molecule is coupled to a fluorochrome, such as fluorescein isothiocyanate (FITC), and added to the chromosomal slide, the probe signal on the chromosome can be visualised by fluorescence microscopy it is possible to build a strong fluorescence signal using several layers of fluorochrome-coupled antibodies.

Directly fluorochromated probes provide an alternative procedure. In Direct labelling of the DNA probes, fluorochromes such as FITC, Cy3, and Cy5 which are coupled directly to the nucleotides (e.g., FITC-12-dUTP) are used.

Denaturation of probe and target DNA

The standard procedures for FISH use air-dried chromosome preparations, which are denatured by heating in 70% formamide at 65°C for 2-3 minutes before being placed in ice-cold 70% ethanol. This is followed by dehydrating through an ethanol series and air drying. The rapid cooling ensures that the

denatured single-stranded DNA in the preparations does not re-anneal. Meanwhile, the DNA probe is denatured for 5-10 minutes at 70°C.

In situ suppression hybridisation

Almost all types of DNA probe used in FISH applications in diagnostic cytogenetics contain interspersed repetitive elements (see section1.2) that will hybridise to complementary sequences throughout the entire genome leading to a high level of background signals. This background can be suppressed effectively by preannealing unlabelled genomic or Cot-I (enriched for highly repetitive sequences) DNA to the labelled probe before hybridising the probe to the target DNA (Landegent e al. 1987, Lichter et al 1988, Pinkel et al 1988). The unlabelled DNA acts as a competitor and will form duplexes rapidly with the repetitive DNA sequences within the probe. The remaining low-copy DNA in the probe will then be available to hybridise with complementary sequences in the target DNA. For hybridisation, the labelled DNA probe is prepared in a hybridisation buffer (containing formamide and dextran sulphate) together with Cot-I DNA. This mix is added to the slide containing denatured target DNA; covered with a coverslip, which is sealed with cow gum; and incubated at 37 to 43°C for 2-16 hours.

Post-hybridisation and detection washings

Following hybridisation, the coverslip is removed, and the slide is washed in 50% formamide and SSC at 42-45°C. The choice of detection method depends on the hapten used in the labelling of the DNA probe. If a biotinylated probe is used, detection involves treating the slide with fluoresceinated avidin and fluoresceinated antiavidin and incubating in detection buffer at 37°C for 30 minutes followed by washing in several changes of detection buffer, SSC with Tween-20, dehydration through an ethanol series, and mounting in DAPI antifade. The slides are viewed using a fluorescence microscope equipped with a UV mercury lamp and appropriate excitation and emission filters. Multiple excitation/dichroic/bandpass filter blocks are available, which can be used in the microscope to observe simultaneously several fluorochromes excited by different wavelengths.

Principles of fluorescence microscopy

Fluorescence microscopy is used to detect structures, molecules or proteins within the cell. Fluorescent molecules absorb light at one wavelength and emit light at another, longer wavelength. When fluorescent molecules absorb a specific absorption wavelength for an electron in a given orbital, the electron rises to a higher energy level (the excited) state. Electrons in this state are unstable and will return to the ground state, releasing energy in the form of light and heat. This emission of energy is fluorescence. Because some energy is lost as heat, the emitted light contains less energy and therefore is a longer wavelength than the absorbed (or excitation) light.

In fluorescence microscopy, a cell is stained with a dye and the dye is illuminated with filtered light at the absorbing wavelength; the light emitted from the dye is viewed through a filter that allows only the emitted wavelength to be seen. The dye glows brightly against a dark background because only the emitted wavelength is allowed to reach the oculars or camera port of the microscope. In epi-illumination excitation light goes through the objective lens and illuminates the object. Light emitted from the specimen is collected by the same objective lens.

Image analysis in FISH

FISH signal tends to fade on exposure to UV light in the fluorescence microscope. This fading can be reduced to a certain extent by the use of antifade agents in the mounting medium. However, the need to detect fluorescent signals of low intensity and their rapid analysis has led to the development of sensitive cameras and computer based image analysis systems. These systems allow the fluorescence image to be digitised and acquired by a computer and archived. The stored image is then available for image processing and analysis. For many applications in diagnostic cytogenetics, however, the DNA probes are large enough to produce bright signals, and image processing is not essential. Digital fluorescence microscopy has particular application in multicolour FISH and in the detection of weak fluorescent signals.

FISH probes and their applications

A wide range of DNA probes are available for chromosome analysis by FISH.

Genomic DNA probes

These include DNA extracted from blood or bone marrow samples, cell cultures or solid tumours labelled by fluorochromes. FISH using genomic DNA probes results in an evenly distributed hybridisation along all the chromosomes. These probes are particularly useful to identify human chromosomes in somatic cell hybrids.

Whole chromosome paint (WCP) probes

These are genomic probes consisting of a cocktail of probes obtained from the different parts of a particular chromosome. These probes are made from chromosome specific genomic libraries, from single chromosome-interspecific somatic cell hybrids, or from flow sorted chromosomes. When this mixture is labelled and used in a hybridisation experiment the entire relevant chromosome is painted. WCP probes are particularly useful for characterising the marker chromosomes and complex chromosomal aberrations. The main disadvantages are that intrachromosomal rearrangements (such as inversions) cannot be identified and that (due to the use of unlabelled Cot-I DNA) centromeric areas and telomeres, are not painted

Centromeric probes

These probes are cloned from repetitive sequences found in and around the centromeric regions of a specific chromosome. All human centromeres except those of chromosomes 13 and 21, and 14 and 22, which share indistinguishable sequences, have chromosome specific sequences of this type. Centromeric probes are suitable for making a rapid diagnosis of one of the common aneuploidy syndromes in interphase nuclei. They are used in aneuploidy detection in uncultured amniotic fluid cells, for preimplantation diagnosis of aneuploidy in cells from blastocytes, for the diagnosis and

detection of residual disease in the management of certain haematological malignancies, and for the analysis of nondisjunctional abnormalities in sperm.

Chromosome-specific unique sequence probes

These are a series of overlapping DNA sequences along each chromosome. These probes are mostly cloned in YAC vectors, which can accept up to 2Mb. Probes cloned in cosmid vectors (up to 40 kb), plasmid vectors (up to 10 kb) and bacteriophage vectors (up to 25 kb) are also available. They are particularly useful for identifying submicroscopic chromosomal aberrations.

Multicolour FISH

Basic principles

The simplest approach to multitarget FISH would be to develop a set of spectrally distinct fluorescent dyes and label each hybridisation probe with a different dye (Nederlof et al 1989). However, this has not yet been achieved.

Fewer fluorescent dyes can be used if more than one dye is used to identify each chromosomal target (Nederlof et al 1990). In combination coding, 2^n-1 targets can be identified, each corresponding to a possible combination of n different fluorescent dyes used in the hybridisation. Different combinations of the three fluorochromes in each probe can give up to seven distinguishable colour differences by digital fluorescence microscopy (Dauwerse et al 1992; Ried et al 1992).

Ratio coding also uses combinations of fluorophores, but varies the relative proportion of each fluorophore used in the combination (Nederlof et al 1992; Dauwerse et al 1992; Lengauer et al 1993). By distinguishing the relative amounts of fluorescence, several targets can be identified for each fluorophore combination used. The simultaneous visualisation of 12 different probes labelled with different fluorescence ratios has been reported. (Ried et al 1992, Lengauer et al 1993, Wiegant et al 1993). Ratio-coded chromosomes can become difficult to distinguish when the number of ratios increases beyond four and ratios of images obtained with a digital camera can be used to better differentiate the targets. This exploits a sensitive

monochromatic, cooled, charged-coupled device (CCD) camera and computerised image processing. A grey-level image of the fluorescence of each fluorochrome is acquired sequentially and merged to provide a false colour. Because the three images must be aligned accurately in the merged image, a system to prevent registration errors, triple band-pass filters are used in the epifluorescence optical.

Fibre-FISH

Chromosome fibres at metaphase are very condensed and therefore the fluorescent signals from two cosmid clones hybridised to the same chromosome can be resolved only if they are more than 2 to 3 Mbs apart. Various techniques have now been developed that release the chromosome fibre from its associated protein within the chromosome scaffold (Fidlerova et al 1994; Parra and Windle, 1993; Wiegant et al 1992). This permits DNA sequences to be hybridised directly onto extended chromosome fibres fixed on a microscope slide and analysed by FISH.

The analysis of extended DNA fibres by FISH has application both in the mapping and ordering of contiguous DNA sequences and, more recently, in the diagnosis of single gene aberrations using cosmid probes. One important example has been in the detection of carriers of intragenic gene deletions in X-linked Duchenne muscular dystrophy (Florijn et al 1995). In this and similar situations, carrier detection by PCR techniques can be difficult, and fibre-FISH can be used.

Reverse chromosome painting

In this technique small numbers of aberrant chromosomes are flow-sorted or microdissected and amplified by DOP-PCR. The products are labelled and used as probes to localise the signal on normal metaphase chromosome spreads (Carter et al 1992). This approach has been used for detailed study of marker chromosomes and to analyse abnormal chromosome with translocations or deletions.

1.4.5 Comparative Genomic Hybridisation (CGH)

Utilisation of FISH for the detection of chromosomal aberrations has depended on the applications of specific nucleic acid probes. Therefore, FISH is generally restricted to a limited number of chromosomal regions. Moreover, pre-knowledge of the regions that might be subject to chromosomal aberration is essential.

CGH is a technique which instead of metaphase cells, utilises a DNA sample from a patient. If the whole human genomic DNA is fluorochrome (or hapten) labelled and hybridised to a normal target metaphase under suppression conditions, all of the chromosomes are stained. However, when certain chromosomes or chromosomal segments are over- or under-represented in the genomic DNA, stronger or weaker signals are expected in the corresponding hybridisation target regions. While large amplifications (>24 copies for an amplicon more than 600 kb) are readily detected based on these principles when there are subtle deviations from a balanced genome, CGH is required.

CGH involves the different labelling of two genomic complement DNAs (test and control) followed by co-hybridisation on normal "target" metaphase chromosomes to assay copy number differences (Kallioniemi et al 1992; du Manoir et al 1993) (Figure 1. 6-1.8). The control DNA introduces an internal standard which prevents errors secondary to experimental parameters which include differential denaturation of chromosomal regions with different base composition, chromatin packaging as well as differential hybridisation of probe sequences due to base composition and target accessibility. Differential fluorescent hybridisation signals represent gains and losses of the test DNA relative to the reference DNA. Ratios of the signals can be quantified along the length of each homologue and provide a copy number karyotype of the test DNA (Figure 1. 7).

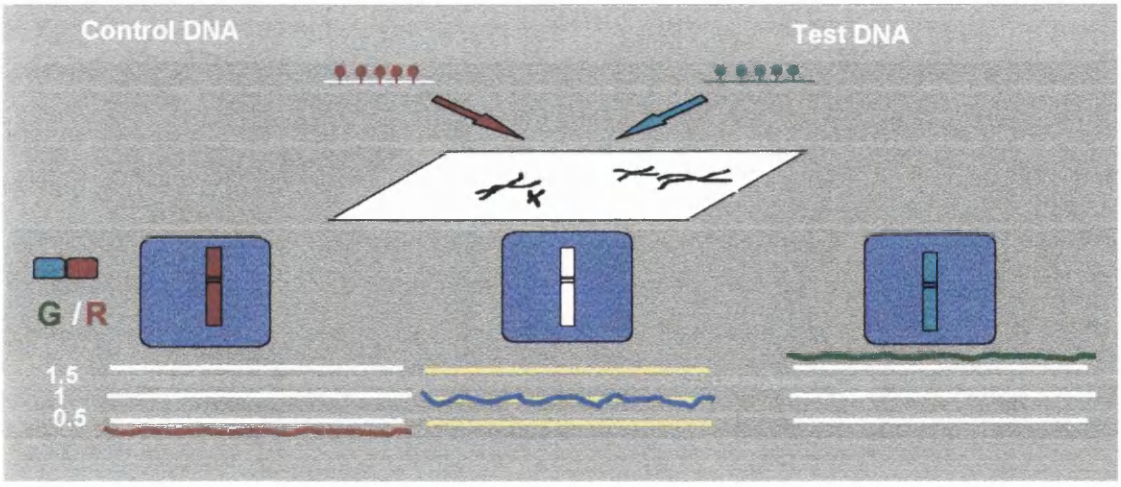


Figure 1. 6: Schematic illustration of CGH. Labelled genomic DNA from test (green) and control (red) are co-hybridised to normal metaphase chromosomes.

Metaphase chromosomes for CGH

Normal metaphase chromosomes are used as hybridisation targets in CGH. The quality of CGH is mainly dependent on the quality of the metaphase chromosomes. It is recommended to prepare a large number of slides at once, so that an entire experiment can be carried out with one batch of slides (Kallioniemi et al 1994). The hybridisation characteristics of all batches of slides are tested using labelled DNAs from a control cell line. In the case that a high quality hybridisation cannot be achieved, the entire batch has to be abandoned. Moreover, slides should contain minimal number of overlapping chromosomes. Preparations with too short chromosomes are also abandoned. After hybridisation chromosomes are counterstained with DAPI to obtain a faint R-banding pattern which helps to identify the chromosomes. Metaphase chromosomes with uniform and adequate hybridisation and good retained morphology are qualified for the analysis (Kallioniemi et al 1994).

Isolation of DNA for CGH

DNA isolated from any tissue can be used for CGH. When CGH is applied on solid tumours, it is very important to ascertain that DNA is isolated from the malignant cells. Contamination of the malignant cells with normal or necrotic cells or regions with inflammations will dilute the tumour DNA and therefore

reduces the ability to see copy number differences. Current methods for DNA isolation yield high-molecular weight DNA suitable for CGH. DNA extracted from formalin-fixed, paraffin-embedded archived tumour specimens has also been used for CGH.

Differential labelling of DNA

Genomic DNA probes for CGH can be labelled either with directly fluorochrome-conjugated deoxynucleotides (direct labelling) or with reporter groups such as biotin or digoxigenin detected via fluorochrome-coupled reporter binding molecules (indirect labelling). Directly fluorochrome-conjugated probes require fewer post-hybridisation manipulations, and improve the quality of hybridisation signal. The size of the DNA before and after labelling is an important consideration in the overall success of the CGH assay. Labelled DNAs should range in size from 500 to 2000 bp to achieve optimal hybridisation. In some cases, very high molecular weight DNA requires mechanical shearing before labelling, and in others the extracted DNA can be labelled without pre-shearing. Nick translation has been the method of choice for labelling the DNAs due to the ability to regulate the nicking activity of DNase I relative to the synthesising activity of DNA polymerase I to achieve optimally sized fragments. Other techniques, such as random priming and degenerate-primer (DOP) PCR have also been used for labelling genomic DNA for CGH (Speicher et al 1993).

Hybridisation and post-hybridisation

Hybridisation protocols essentially follow those used in FISH (section 1.4.4). Equal amounts (120-500 ng) of each of the differentially labelled test and control DNAs are mixed with human Cot-I DNA (2-10 μg), ethanol precipitated, and dissolved in the hybridisation buffer. Cot-I is added to block the binding of the labelled repetitive sequences in both genomes. Repeat sequences, if not adequately blocked, tend to result in large ratio changes at the peri-centromeric and heterochromatic regions. The hybridisation mix is then heat-denatured and allowed to preanneal to compete out hybridisation signal due to repeat sequences. Before hybridisation, the slides containing

the target metaphases are denatured. The chromosomes require sufficient denaturation to permit accessibility of the labelled DNA, however, the chromosomal morphology must be retained for identification purposes. Denaturation is carried out at 70-74 °C for 2-3 minutes in 70% formamide/2 x SSC followed by (in some protocols) a proteinase K digestion. In situ hybridisation is carried out in a moist chamber at 37°C for 2-4 days. Post-hybridisation steps are carried out as in standard FISH. In case of indirect labelling of the genomic probes, immunological detection with appropriate fluorochrome coupled reagents is carried out. After the appropriate detection steps, the chromosomes are DAPI counterstained for identification purposes.

Fluorescence microscopy and visual analysis of CGH

Visualisation of the fluorescent hybridisation signal is performed with a fluorescence microscope using appropriate double or triple band pass filters that allow simultaneous visualisation of two or more fluorochromes. A rough assessment of the quality of the hybridisation experiment can be made according to the background levels and the differences in the fluorescence intensity in the sex chromosomes (when test and control DNAs are derived from different sexes). For visual analysis, the intensity of two fluorescent dyes must be compared along the length of a chromosome where similar hybridisation signals should be observed for each homologue. Gains of DNA sequences in the test DNA relative to the control DNA are visualised as increased fluorescent signals of the test DNA on the chromosomal region from which the sequences are derived. Losses are detected as an increased relative fluorescent signal of control DNA. High level gene amplifications (>10-20 fold) as well as large gains and losses affecting chromosome regions are visually detectable. Small deletions and other lower level copy number differences and ratio changes in tumour cells contaminated with normal cells often cannot be reliably evaluated without digital image analysis (Kallioniemi et al 1992, 1994; du Manoir 1993).

Acquisition and display of digital images

For a comprehensive assessment of all chromosomal imbalances present in the test DNA, quantitative measurement and analysis of fluorescence intensities are necessary. The acquisition of image is carried out by first carefully scanning the entire slide for the best metaphase cells. A sensitive (preferably cooled) CCD camera is required to obtain digitised metaphase images. Grey level images of each of the fluorochromes are obtained using a computer-controlled filter-wheel with a series of single-band-pass excitation filters which sequentially excite DAPI, FITC and rhodamine or TexasRed. This allows visualisation of all three fluorochromes without any registration shifts between images (Figure 1. 8). After an optimum optical as well as exposure time settings is worked out, it should be kept constant for a series of metaphases corresponding to a single case. In general, using a cooled CCD camera, exposure times are in the range of 1-10 seconds for FITC and 0.5 to 2 seconds for rhodamine or TexasRed images.

Quantitative analysis of green and red fluorescence ratio profiles for CGH

The three fluorochrome-specific high-resolution images captured from each entire metaphase spread with CCD camera are stored in an appropriate format and used for the analysis. Various software in different computer platforms are available for the CGH analysis.

Evaluation of CGH images

The quality of hybridisation is critically important for the reliable CGH analysis. Image and hybridisation quality can be assessed either visually, or by objective criteria defined by image analysis. All images obviously should be well focused and show smooth high intensity hybridisation. In case of indirect labelling granulation is the most common problem leading to high noise background. Good quality hybridisations are uniform rather than granular. Metaphases that contain high degrees of non-specific granules and fluorescence spots on the chromosomes are not used for CGH analysis. Analysis of chromosomes with banding pattern of fluorescence signals which

are not adequately denatured is unreliable. On the other hand, over-denatured chromosomes with damaged morphology also are not suitable for CGH analysis. Distribution of the green and red fluorescence should be similar between the sister chromatids of a chromosome, the two chromosome homologues in each metaphase and the same chromosome between different metaphases. Distribution of the background fluorescence on the slide surrounding the chromosome should be minimal and uniform. Fluorescence intensity corresponding to the test and the control DNA should be minimal at the centromeric regions. Finally, chromosomes should show adequate length with intense DAPI banding pattern and minimal overlapping.

Initial chromosome segmentation

After selecting suitable CGH images, the chromosomes are segmented automatically using the "chromosome definition image" obtained by adding the counterstain reference images after standardising them so that they have the same range of pixel values.

The two images are combined, because some reports suggest that segmentation based on a DAPI counterstain image alone is inadequate due to weak staining in some regions particularly chromosome telomeres.

Background correction

Background fluorescence must be removed before the fluorescence ratios are calculated. Background can be removed by subtracting the intrachromosomal fluorescence intensity from the chromosomal fluorescence. If background varies across the metaphase, a local estimate is required for each chromosome

Normalisation of fluorescence intensities

Normalisation of fluorescence intensities is necessary for meaningful comparison of data from different metaphase spreads and different preparations. Normalisation minimises the effects of differences in the hybridisation and imaging conditions. Two main approaches are currently used for normalisation. In the first, the total intensities of the metaphase

images resulting from the two hybridisations are made equal. In the second approach, the computed ratio profiles are normalised so that the modal ratio profile value for a metaphase is one.

Segmentation correction

After background removal and normalisation of the fluorescence intensities, minor errors in the segmentation of chromosomes are corrected interactively. This stage is dependent on the type of the software used and aims to separate touching chromosomes, cut out non-target areas of the image, like interphase nuclei, or exclude the chromosome clusters which are not separable from the analysis.

Green to red ratio profiles.

At this stage, for each (segmented) chromosome, the symmetry axis is determined and green to red ratio are integrated along lines that are perpendicular to the medial axis and spaced at one pixel intervals along the axis. The integration covers both chromatids and thereby comprises the sum of two independent observations. The integrated green to red ratio profiles that run from the terminal region of the short arm (pter) to the terminal part of the long arm (qter) of each chromosome are then used to calculate the green /red ratio profile for each chromosome.

Averaging of profiles collected from several metaphases

To reduce the effects of random noise in CGH images, data from 5-20 metaphase images (10-40 chromosomes) are combined and profiles of the mean ratio and $\pm$ 1 SD are calculated and displayed. To (at least partly) compensate the effect of different and/or non-uniformly condensation, all ratio profiles are normalised to a standard length for each chromosome type.

Sensitivity and Mapping resolution

It has been shown that a linear correlation between chromosome copy number (1-5) and the average green to red ratio is detectable. This relationship is only obtained when the size of the affected region is in the

range of 10-20 Mb (Kallioniemi et al 1992). It is predicted that this can be improved up to 3-5 Mb which is the smallest distance that can be adequately discriminated by FISH. Detection of copy number aberrations of smaller sizes is possible if the DNA sequences show high level of amplification. The total amount of the amplified DNA required to be detectable by CGH is estimated to be at least 2Mb (Piper et al 1994).

Applications of CGH

CGH was developed to be used in tumour cytogenetics where banded chromosome analysis is often difficult, or even impossible. Since its development, CGH has been applied mostly as a useful tool for research tumour cytogenetics with more than 300 publications since 1992. CGH studies have resulted in a large set of new data regarding recurrent chromosomal aberrations in solid tumours. The recurrent genomic alterations detected by CGH have lead to the localisation of the genes relevant for the development of certain tumours (Mohamed et al 1993; Kallioniemi et al 1994, 1995; Speicher et al 1994; Reid et al 1995). CGH has become an important method for the detection of proto-oncogenes, which are amplified in certain tumours.

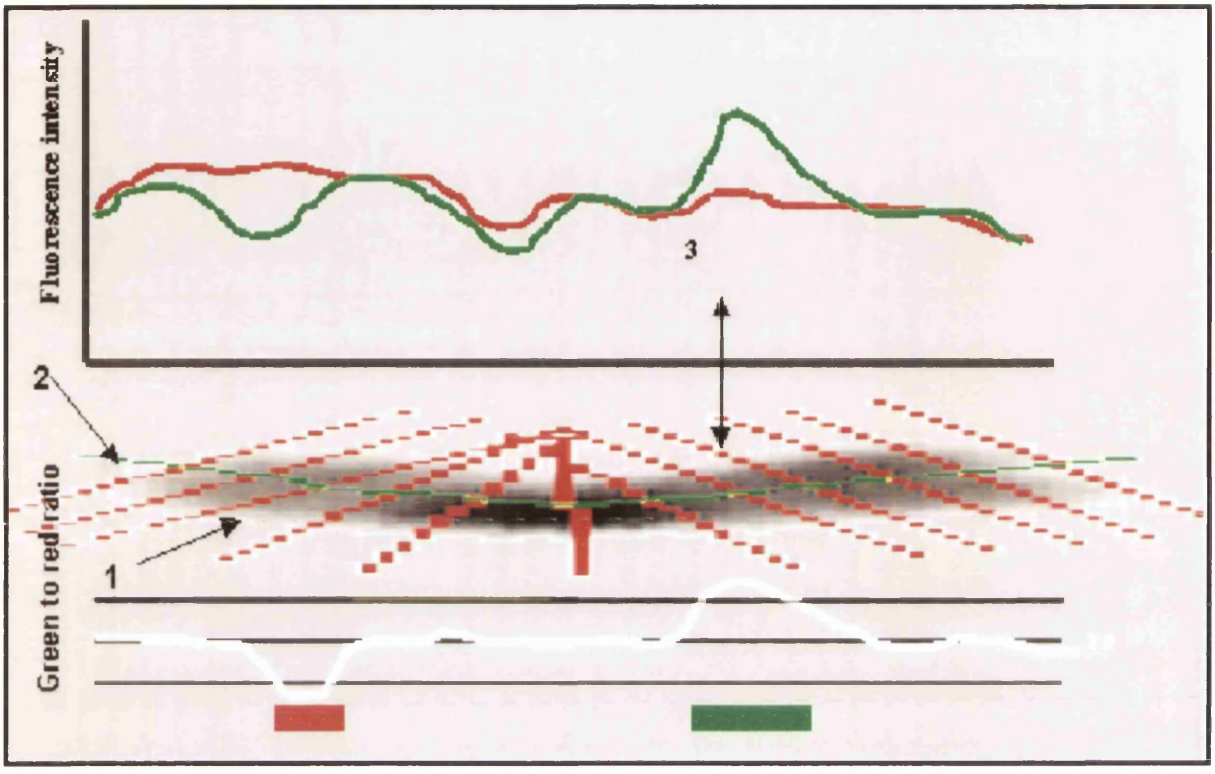


Figure 1. 7: A schematic diagram of CGH analysis illustrating the quantitation of green and red fluorescence intensity ratios along chromosomes by a digital image analysis program. (a) Chromosomes are first segmented and the chromosome outline (1) is displayed. The medial axis (2) is determined based on this outline. Green and red fluorescence intensities are then integrated along transverse lines (3) drawn across the medial axis at one-pixel intervals from p-telomere to q-telomere. Quantitation is performed from two separately collected gray-level images of the metaphase spread in both green and red fluorescence. Integrated green and red fluorescence intensities and their ratios are determined for each pixel along the medial axis and plotted as a histogram. The ratios are normalised so that the average ratio for the entire metaphase spread is 1.0. Chromosomal regions that show an increased green to red ratio are present at an elevated copy number (gained, amplified) in the test DNA, whereas those with a decreased ratio are lost from the test DNA (red thick line).

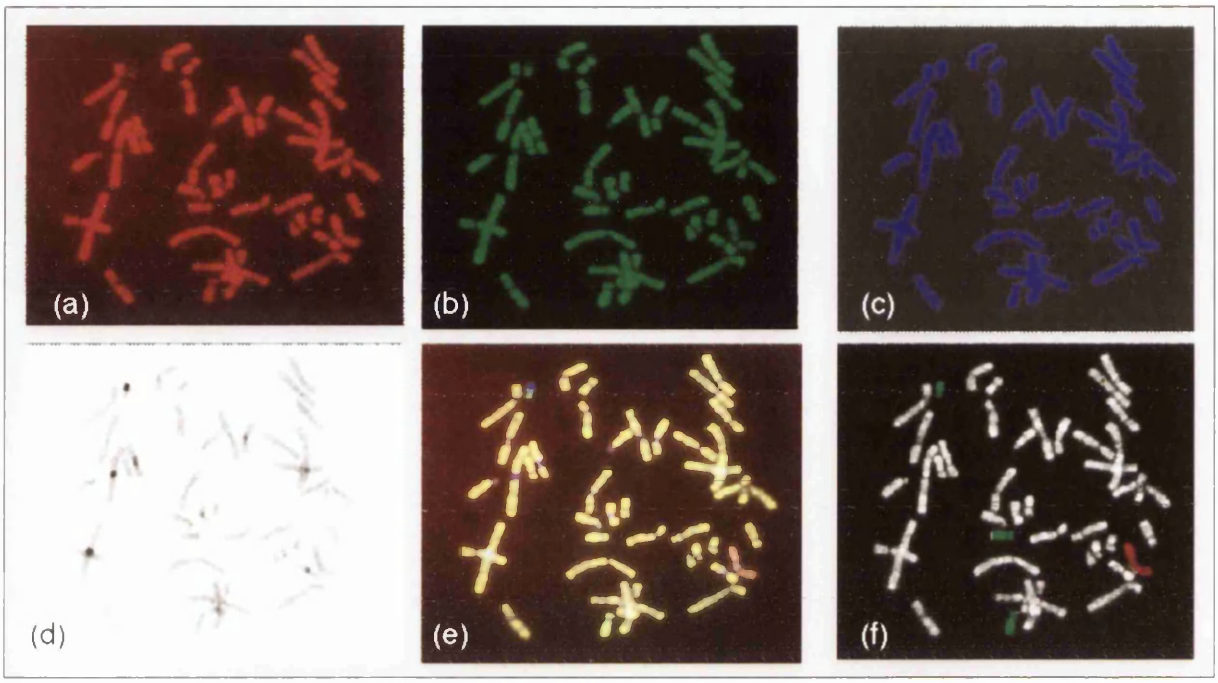


Figure 1. 8: Detection of chromosomal imbalance by CGH and digital image analysis using DNA samples from a male patient with trisomy 18 (test DNA) and a normal female individual (control DNA) (a) Hybridisation patterns of TexasRed labelled (control) and (b) FITC-labelled (test) DNA. (c) DAPI counterstain image of the target metaphase. (d) Digital conversion of the image shown in c providing a banding pattern resembling classic G-banding, thus facilitating identification of the chromosomes. (e) Mixed colour image obtained by digital overlaying of the images shown in a and b. Greenish/reddish colours indicate chromosomal sequences that are overrepresented/underrepresented in the test DNA compared to those in the control DNA. (f) Global CGH analysis. This image shows a transformational of the intensity ratio profile into three colours. Gains are indicated in green, losses are indicated in red, and the normal chromosomal complements is indicated in white.

CGH has detected many previously unknown abnormalities in various types of solid tumours. Chromosomal copy number changes in breast carcinomas, small cell lung carcinomas (SCLC), malignant gliomas, and uveal melanomas were detected very early after the development of the CGH. Most of the gains identified by CGH were novel and included frequently observed high level amplifications. These amplifications are likely to indicate the chromosomal regions, where genes relevant for tumour development or progression are localised. These developments will probably lead to inclusion of DNA probes and probe sets in analysis of the corresponding tumour type by other methods such as interphase cytogenetics. Comparison of G-banding with CGH results in leukaemias has revealed discrepancies in a large number of cases. CGH is also used in combination with DOP-PCR to analyse DNA gain or losses in samples containing a very few cells which are not sufficient for conventional techniques. Recent applications of CGH in haematological malignancies and clinical cytogenetics are discussed together with the results of this study in chapter 4.

Limitations and difficulties

CGH cannot detect balanced translocations or inversions. Point mutations and small intragenic abnormalities also are not detectable by CGH. Peri-centromeric and heterchromatic regions cannot be reliably analysed as they are being blocked by competitive DNA (Cot-I) in hybridisation, although in cases of malignancy this problem can be partly overcome by using the control DNA from the normal tissue of the same individual.

Contamination of the tumour DNA with surrounding normal tissues will dilute the test to control ratio changes. This causes serious difficulties when the normal tissue contribution is more than 50%. In bi- or multi-clonal tumours, the different genetic aberrations present in individual clones may sometimes compensate one another or exist at too low a frequency to be detected.

There are some reports that when the biotin labelled test DNA is compared with digoxigenin labelled control DNA and detected by avidin-FITC and anti-digoxigenin rhodamine, the green to red ratio at 1p32-pter, 16p, 19 and 22 may occasionally be distinctly below the average ratio. This would therefore

lead to a false positive interpretation of a deletion. This problem may be due to differential hybridisation properties of digoxigenin and biotin labelled probes. This problem is not reported using directly labelled probes (du Manoir et al 1993; Kallioniemi et al 1994).

Due to normalisation steps in the CGH image analysis, ploidy changes are not independently detectable in CGH. CGH has not been used as a diagnostic tool in any clinical genetics laboratory, nor has it been used for the detection of constitutional chromosome abnormalities. Moreover as CGH uses normal metaphase chromosome as target DNA, its resolution is limited to that of routine cytogenetics. Furthermore CGH follows 48-72 hours protocols, which makes it impossible to use in urgent situations. Finally, as the fluorescence intensity corresponding to the test and control DNA gradually decrease at the chromosome ends, when the absolute intensities approaches the background fluorescence, unreliable ratio changes may appear and therefore current approaches to CGH image analysis are unable to analyse the telomeric regions reliably.

1.5 Aims of this study

The aims of this study were first, to optimise the comparative genomic hybridisation technique and test its reliability as a diagnostic tool in a clinical genetics laboratory, second, to investigate whether any of the current limitations could be overcome, and third, to improve the resolution of the technique beyond the current limits by targeting DNA molecules instead of metaphase chromosomes.

2: Materials and Methods

2.1 Safety

Lab coat and gloves were worn at all times when handling blood and other biological or chemical materials, which were always handled in appropriate category safety cabinets. All glassware, most plastics and all tips for micropipetting were sterilised in an autoclave (Denly) at 121°C for 15 minutes. When necessary, solutions were also sterilised by autoclaving.

2.2 Patients and samples

The clinical cytogenetics service in this department serves a population of roughly three million people. Samples used in the early stages of this study had been sent for diagnostic proposes, and if there was enough material left, then it was used for the work described here.

Blood was the most frequently used tissue specimen in this study. Blood samples from normal male individuals were used for preparation of normal target metaphases in CGH, and samples from patients were used for targets in FISH and for conventional cytogenetic studies. Genomic DNA extracted from blood specimens of patients and normal individuals was used as test and control DNA in CGH experiments. Bone marrow and solid tumour samples also were used in CGH, FISH and conventional cytogenetic experiments.

After optimisation, CGH was first used in patients with constitutional chromosome abnormalities, solid tumours or haematological malignancies to compare its capabilities with conventional methods. CGH investigations then were performed on samples from patients with haematological malignancies or solid tumours when conventional techniques had failed to resolve the abnormalities.

For detection of cryptic chromosome abnormalities, families with two affected members with apparently normal karyotype and a similar phenotype related through at least one healthy first degree relative were selected. These features are highly suggestive of the segregation of a cryptic chromosomal translocation.

2.3 Culture techniques and chromosome preparation

2.3.1 Peripheral blood culture

Solutions:

Growth medium	
Iscoves medium	100 ml
Heparin	1 ml
Glutamine	1 ml
PHA	2 ml
Penicillin/Streptomycin	1 ml
(100 units/ml and 100 μg/ml respectively)	

Procedure:

Using an aseptic technique, 0.5 ml heparinised whole blood was added to 4.5 ml of growth medium and mixed by gently inverting each culture tube a few times. The culture tubes were then incubated in a slanting position at 37°C for 72 hours.

2.3.2 Harvesting

Solutions:

Colcemid solution:	10 μg/ml
Hypotonic solution:	0.075 M KCl
Fixative solution:	3/1 methanol/acetic acid

Procedure:

0.1 ml Colcemid solution was added to each 5 ml culture, mixed gently by shaking the tube and incubated at 37°C for an additional 45 minutes. The tubes were then spun at 1200 rpm for 8 minutes before pipetting off the supernatant into a container of 10% Chloros. The pellet was resuspended in 10 ml of hypotonic solution and incubated for a further 7 minutes. The tubes were then spun at 1200 rpm for 8 minutes and the supernatant was pipetted off into a container of 10% Chloros. The pellet was carefully resuspended in 10 ml of fresh fixative and spun at 1200 rpm for 8 minutes before pipetting off

the supernatant. Once again, the pellet was resuspended in fresh fixative and spun for 8 minutes before pipetting off the supernatant. The pellet was then thoroughly resuspended in 5 ml of fresh fixative and placed in refrigerator for at least 30 minutes.

2.3.3 Slide preparations

Microscope slides were cleaned by soaking in Decon® overnight and rinsed with running tap water for at least 2 hours. The clean slides were then stored in water for at least 30 minutes at +4°C before use. The tubes containing the cell suspension were removed from the refrigerator and spun at 1200 rpm for 8 minutes. After pipetting off the supernatant, the pellets were resuspended in 0.5 ml of fresh cold fixative solution. 3-4 drops of the cell suspension were then dropped onto a cold, wet slide. For G-banding, the slides were then placed on a hotplate to dry and evaluated under a light microscope (Phase contrast). Slides which passed the quality control were then baked for at least one hour at 80°C before use.

2.3.4 G-banding

Solutions:

Trypsin solution:	
Difco trypsin	1 vial
Saline solution	10 mls
2 x SSC (pH 7.4):	
NaCl	17.50 g
Na citrate	8.82 g
Distilled water	up to 1000 mls
Staining solution:	
Leishman powder	3 g
Methanol	up to 2000 mls
(For staining slides this solution was diluted with buffer (pH 6.8) in the ratio 1 part to 3 parts buffer)	
Saline solution:	0.9 g/L NaCl

Procedure:

The giemsa bands (G-bands) were obtained by digesting the chromosomes with the proteolytic enzyme trypsin. The aged slides were immersed in trypsin solution for 7-15 seconds and then rinsed with saline solution before being stained in Leishmanns solution for 2 minutes. The rinsed slides were then placed on a hotplate to dry and analysed under a light microscope.

2.4 Fluorescence *in situ* hybridisation (FISH)

Uni-colour or multi-colour targeted FISH using painting, centromeric or telomeric probes was carried out mostly to confirm the Comparative genomic hybridisation (CGH) results. In families where CGH had already detected the abnormality, targeted FISH was used to detect carriers or other affected individuals in the family. In most circumstances, commercially available labelled probes were used, in some cases, however, fluorochrome or hapten labelling of the probes was performed in the laboratory.

2.4.1 Labelling of probes by nick translation

Both direct and indirect labelling of DNA probes were performed in this study.

Solutions and reagents:

Enzymes

DNA polymerase I/DNase (0.5U/µl)/(40 pg/µl) — Gibco BRL

10x A4 Solution (Gibco BRL)

0.5 M Tris-HCl, pH 8.0
50 mM $MgCl_2$
0.1 mg/ml bovine serum albumin
0.1 M ß-mercaptoethanol
0.2 mM dATP
0.2 mM dCTP
0.2 mM dGTP

Reporter Molecules:

Either: 40 µM Fluorescein-12-dUTP (DuPont) or
40 µM TexasRed-5-dUTP (DuPont) or
40 µM Biotin-11-dUTP or
0.125 mM digoxigenin-11-dUTP

DNA precipitation solutions:

Sodium acetate — 3 M
Ethanol — 100%

TE buffer:

10 mM Tris/HCl pH 8.0 ; 1 mM EDTA

Distilled water

Direct labelling of DNA

For nick translation a final volume of 50 μl solution containing 1 μg of the DNA to be labelled, 5 μl DNA polymerase I/DNase, 5 μl of 10xA4 solution and either 40 μM Fluorescein-12-dUTP, or 40 μM TexasRed-5-dUTP dissolved in distilled water was incubated for 90 minutes at 15°C in a water bath. After nick translation, the reaction was stopped by incubating the reaction tube in a waterbath at 70°C for 10 minutes. 2.5 μl sodium acetate and 150 μl ethanol was then added to the reaction tube. The labelled DNA was precipitated by vortexing the tube for 30 minutes at 14000 rpm. After pouring off the supernatant the DNA pellet was resuspended in appropriate amount of TE buffer.

Indirect labelling of DNA

In this method, digoxigenin or biotin were used as reporters instead of directly fluorochrome coupled nucleotides. Biotinylation was carried out using Biotin-11-dUTP in exactly the same manner as the previous method. For digoxigenation of the DNA probes 0.125 mM digoxigenin-11-dUTP was used.

2.4.2 Labelling of probes by PCR

Solutions and reagents:

DNA polymerase (Amplitaq®)	Perkin Elmer
10x reaction buffer	Perkin Elmer
dATP (10 mM)	
dCTP (10 mM)	
dCGP (10 mM)	
dTTP (1.2 mM)	
Primers (forward and reverse) 20uM each	
FITC-12-dUTP (1 mM)	DuPont
TexasRed (1 mM)	DuPont
Biotin-11-dUTP (0.4 mM)	
Distilled water	

Procedure:

1 μg of genomic DNA or 1-2 ng of the target DNA probe (where probe was in plasmid, cosmid or YAC) was used for PCR DNA labelling.

PCRs were performed in a total volume of 50 μl. (0.5 μl of AmpliTaq® DNA polymerase, 1 μl of 10x reaction buffer, 1 μl of each dATP, dCTP, dGTP, 2.5 μl dTTP, 5 μl of each primer, 5 μl of FITC-12-dUTP or TexasRed-5-dUTP, or 2.5 μl Biotin-11- dUTP 0.4 mM, appropriate amount of the DNA to be amplified, and up to 50 μl distilled water).

Fifty microlitres of mineral oil was added on top of the reaction mixture to prevent evaporation. PCR reactions were performed as described in Table 2. 1. The reaction products then were purified by ethanol precipitation or by using a Millipore Ultrafree ® -MC filter.

Table 2. 1: PCR program for labelling of DNA probes

1 cycle:	
94 °C	2 minutes
50°C	1 minute
72°C	1 minute
30 cycles:	
94°C	1 minute
50°C	1 minute
72°C	1 minute

2.4.3 Pre-hybridisation and hybridisation

Materials, reagents, and solutions

Glass slides with metaphase chromosomes	
Labelled DNA probes	
Competitor DNA	Cot-I DNA (BRL Life Sciences)
Cold ethanol	70%, 90%,100%
Deionised formamide	
Hybridisation solution: sulphate (Pharmacia), 1 x SSC	50% formamide, 10% dextran,
1 x SSC.	1.5 M NaCl, 15 mM Na_3-Citrate
Denaturation solution:	70% formamide, 2 x SSC pH 7.0
Rubber cement	
Water baths	37°C, 45°C, 60°C, 70°C

Slide preparation

The metaphase slides were examined under a light microscope (phase contrast) and positions of metaphases of acceptable quality were recorded by marking a 2 cm^2 slide area appropriate for hybridisation. Slides were then dehydrated through an ethanol series (2 minutes each at 70%, 90% and 100%) and used after 1-2 days. Where urgent results were required, the slides were aged by incubating at 60-80°C for one hour immediately after they had dried at room temperature.

Denaturing of target DNA and the probe

Denaturing of target DNA and the probe were performed in parallel. Slides were denatured by incubating in 70% formamide (v/v) in 2 x SSC at 70°C for 2 minutes. After denaturation, the slides were quenched in ice-cold 70% (v/v) ethanol and then dehydrated in ethanol series. Ten microlitres of hybridisation solution containing 10-40 ng of labelled test and 1 μg of unlabelled human Cot-I DNA were denatured by incubating at 65°C for 10 minutes. The tube containing the denatured probe was then immediately transferred into a 37°C water bath and incubated for 30 minutes to allow

CISS hybridisation to occur. In dual colour FISH the two probes were mixed in equal amount before they were denatured.

Hybridisation

Slides containing the denatured target metaphases were transferred onto a metal tray and 10 µl of the hybridisation solution was applied to the marked area which was then covered with a coverslip, and the edges were sealed with rubber cement to prevent evaporation of hybridisation solution. Finally, the probe(s) and the target DNA were allowed to hybridise at 37-42°C overnight, in a metal tray floating in a water bath.

2.4.4 Post-hybridisation

Solutions, materials and reagents

1 x SSC.	1.5 M NaCl, 15 mM Na_3-Citrate
2 x SSC	
1 x ST solution:	4x SSC, 0.05% Tween-20
Washing solution A	50% formamide, 2 x SSC
Blocking agent:	15% human serum in ST solution
Detection agents:	Avidin -FITC
	Antidigoxigenin – rhodamine
Counterstain:	0.1 µg/ml DAPI (4,6,diamino-2-phenylindole)/ Antifade (Citiflour)
Alcohol series	70%, 90%, 100% ethanol
Coplin jars	
Water baths	37°C, 42°C

Washing steps

The slides were taken out from the water bath before the rubber cement was removed. The coverslips were rinsed off by incubating the slides in 2 x SSC for 5 minutes. Following that, the slides were washed 3x5 minutes in solution A at 42°C, 1 x 5minutes in 1 x SSC at 42°C followed by 1 x 5minute in 1 x ST at 42°C and 1 x 5 minute in 1 x ST at room temperature. Where directly labelled probes had been used the slides were dehydrated in an alcohol

series and counterstained as will be described later. When using biotin or digoxigenin labelled probes, the slides underwent signal detection and amplification steps.

Detection

Slides hybridised with digoxigenin or biotin labelled probes were incubated for 10 minutes with 100 μl blocking agent at 37°C. Slides were then incubated with avidin-FITC conjugate for 30 minutes at 37°C to visualise biotin labelled probes. Digoxigenin-labelled probes were detected by incubation with sheep anti-digoxigenin conjugated to rhodamine. In dual colour experiments both digoxigenin and biotin labelled probes were detected at the same time. No amplifications of the signals were required. Slides were then washed 3×5 minutes in ST solution at room temperature, dehydrated through an alcohol series and then air dried. Finally, they were counterstained by DAPI, resulting in coarse banding of the chromosomes, allowing individual chromosomes to be visualised and identified.

Visualisation

Observation of signals was carried out by either directly by using the 100x/1.30 oil objective of an epifluorescence microscope (Zeiss Axioplan) equipped with specific filter sets, or by means of a cooled CCD camera and computer (Apple Macintosh Quadra 950) with SmartCapture software fitted to the same microscope. The photographs taken were printed with a full colour printer connected to the system (Mitsubishi).

2.5 Extraction of genomic DNA

2.5.1 Blood and bone marrow

Solutions:

RBC Lysis:

155 mM Ammonium Chloride

10 mM Potassium Hydrogen Carbonate

1 mM EDTA

Cell Lysis:

25 mM EDTA

2% SDS

Proteinase K: (20mg/ml)

Protein Precipitation:

10 mM Ammonium Acetate

Isopropanol

Ethanol (70% v/v)

TE buffer

10 mM Tris/HCl pH 8.0

1 mM EDTA

Procedure:

One volume whole blood or bone marrow sample collected in EDTA was added to 3 volumes cold RBC lysis solution. The sample tube was then inverted to mix and kept at room temperature for 20 minutes before being centrifuged at 2000g for 7 minutes (large samples) or 20 seconds (small samples). The supernatant was then removed leaving a visible white cell pellet and 10-20 µl of residual liquid. The sample tube was vortexed to re-suspend cell pellet before adding 1 volume cell lysis solution and vortexing again. 1/3 volume protein precipitation solution was added and vortexed before centrifuging at 3000g for 20 minutes to pellet proteins (3 minutes in microfuge for small samples). Supernatant was then transferred to a clean tube containing 1 volume isopropanol and mixed thoroughly to form DNA precipitate. DNA was then spooled out using sealed Pasteur pipette, and washed in 70% ethanol. For small samples the DNA was spun down. The isolated DNA was then dissolved in appropriate volume of TE buffer.

2.5.2 Fixed cells

For isolation of DNA from cells fixed in methanol/acetic acid, the fixative was removed by washing the samples twice in 2 x SSC. For this: 5 ml 2 x SSC was added on top of the existing fixative in the sample tube and spun for seven minutes. After removing the supernatant a further 5 ml of 2 x SSC was added and the tubes spun for another 7 minutes. After removing the supernatant 5 ml of cell lysis buffer was added and the DNA extracted as described for blood and bone marrow samples.

2.5.3 Solid tumours

10-20 mg of fresh or frozen tissue was added to a 1.5 ml tube containing 600 µl cell lysis solution chilled on ice and quickly homogenised using 30-50 strokes with microfuge tube pestle. The homogenised sample was then incubated in a water bath at 65°C for 55 minutes. 3 µl Proteinase K solution (20mg/ml) was then added and the lysate was incubated at 55°C for 3 hours to overnight, or until tissue had dissolved. 3 µl RNase solution was then added to the cell lysate, mixed by inverting the tube, and incubated at 37°C for 30 minutes. DNA isolation then was carried out as described for blood and bone marrow samples.

2.6 Comparative Genomic Hybridisation (CGH)

2.6.1 Metaphase Spreads

Metaphase spreads were prepared as described in 2.3. Wet slides were left at room temperature to dry and used after 1-2 days. Where urgent results were required, the slides were aged by incubating at 60-80 °C for one hour immediately after they had dried at room temperature

To minimise inter-experimental variability in CGH preparations, batches of metaphase slides comprising 100-200 slides were prepared at once. Several slides from each batch were then examined for high quality chromosomes. Batches of slides with high quality metaphases and minimal overlapping chromosomes were selected and their hybridisation characteristics tested in

control experiments with 1:1 mixtures of differentially labelled normal genomic DNAs. Suitability of slides was also predicted based on the intensity of DAPI signal arising from interphase and metaphase chromosomes after denaturing (for details see Results). Each batch was used for future experiments only if high quality hybridisation had been achieved in control CGH experiments or it had passed the DAPI intensity evaluation procedures (see Results). Otherwise, the entire batch was abandoned.

2.6.2 Genomic DNA Probes and Labelling Procedures

Test and control DNAs were extracted as described above. Control genomic DNAs were prepared from the blood of healthy males (46,XY) or females (46,XX). Test (patient) human genomic DNA was directly labelled with FITC-12-dUTP (DuPont), and control DNA was labelled with TexasRed-5-dUTP (DuPont) by standard nick translation reaction (2.4.1). DNase I concentration was adjusted to result in an average fragment size of 500-1000 bp.

2.6.3 Pre-hybridisation and hybridisation

Solutions and reagents

Labelled DNA probes

Competitor DNA:

Cot-I DNA (BRL Life Sciences)

Cold ethanol:

70%, 90%,100% (v/v)

Deionised formamide

Hybridisation solution:

50% formamide, 10% dextran sulphate (Pharmacia), 1 x SSC

1 x SSC:

1.5 M NaCl

15 mM Na_3-Citrate

Denaturation solution:

70% formamide, 2 x SSC pH 7.0

Procedure:

Metaphase chromosomes from normal male individuals were denatured for 2 minutes at 70°C in 70% formamide, 2×SSC pH 7.0; thereafter, slides were put through an ice-cold ethanol series (70%, 90%, 100%) and air-dried. For each hybridisation area, one microgram of labelled test DNA (5 μg in overnight CGH), 1 μg of labelled control DNA (5 μg in overnight CGH) and 50 μg of unlabelled human Cot-I DNA were dissolved in 10 μl of hybridisation solution and denatured at 74°C for 7 minutes. The hybridisation mix was then applied to the slide with the denatured metaphase chromosomes, covered by an 18×18 mm coverslip and sealed with rubber cement. Finally, the genomic DNA probes and the target DNA were allowed to hybridise at 37°C for 48-72 hours in a metal tray floating in a water bath.

2.6.4 Washing steps

Solutions, materials and reagents

1 x SSC	
	1.5 M NaCl
	15 mM Na_3-Citrate
2 x SSC	
1 x ST solution:	
	4x SSC
	0.05% Tween-20
Washing solution A:	
	50% formamide, 2 x SSC
Counterstain:	
	0.1 μg/ml DAPI (4,6,diamino-2-phenylindole)/ Antifade (Citiflour)
Alcohol series:	70%, 90%,100% ethanol

Procedure

After hybridisation at 37°C, slides were washed 3×5 minutes with 50% formamide and 5 minutes with 2×SSC at 42°C and another 5 minutes with 1 x ST solution at room temperature. Slides were then dehydrated in an ethanol series (70%, 90%, 100%) and air dried. Finally, they were

counterstained with DAPI resulting in coarse banding of the chromosomes, allowing individual chromosomes to be visualised and identified.

2.6.5 Digital image analysis

Images were obtained using an epifluorescence microscope (Axioplan: Zeiss, Germany) equipped with a cooled CCD camera (Photometrics) controlled by an image analysis system.

For normalisation of the ratio profiles, the modal value of the green-to-red ratio for the entire metaphase was set to 1.0. Finally, the individual ratio profiles were combined to yield the average ratio profiles, which were displayed next to the chromosome diagrams with significance intervals of 0.5 and 1.5.

Chromosomal regions with a green-to-red ratio above 1.5 were considered to be over-represented (gained), whereas regions with a ratio below 0.5 were considered to be under-represented (lost). These limit values were slightly different in each experiment depending on the thresholds deduced from the analysis of negative control experiments where two differently labelled normal DNAs were hybridised against one another.

Standard CGH analysis was performed using either SmartCapture V 2.1 software (Digital Scientific, Cambridge, UK) or recently, the Quips programme for CGH analysis.

2.6.6 SmartCapture™ V 2.1

CGH analysis using SmartCapture included flat fielding, background removal, image enhancement, thresholding and fluorescence equalisation followed by global analysis, axial analysis, scoring and the final analysis.

Image capture

Using DAPI filter of the fluorescence microscope one high-intensity, uniformly hybridised metaphase cell was localised. **Capture** was selected from the

SmartCapture extensions menu and Capture, Camera and Filter parameters were set as shown in Figure 2. 2.

By clicking on the **Expose** button, the settings were accepted and the image was captured. Three excitation frequencies, red (TexasRed), green (FITC) and blue (DAPI) were seen consecutively. The image composed itself of three colour components and after a brief pause, the complete colour picture together with a colour normalisaton window was displayed (Figure 2. 1).

The image then was saved as Macintosh PICT format in a folder designated for a particular case. For each CGH experiment between 5-20 images were captured. Normalisation settings were kept constant for the second image onward.

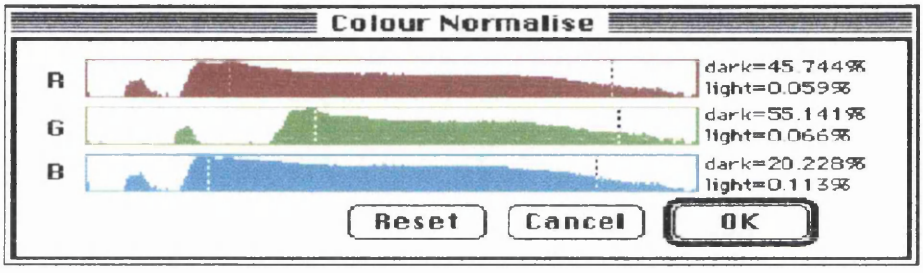


Figure 2. 1: Normalisation window

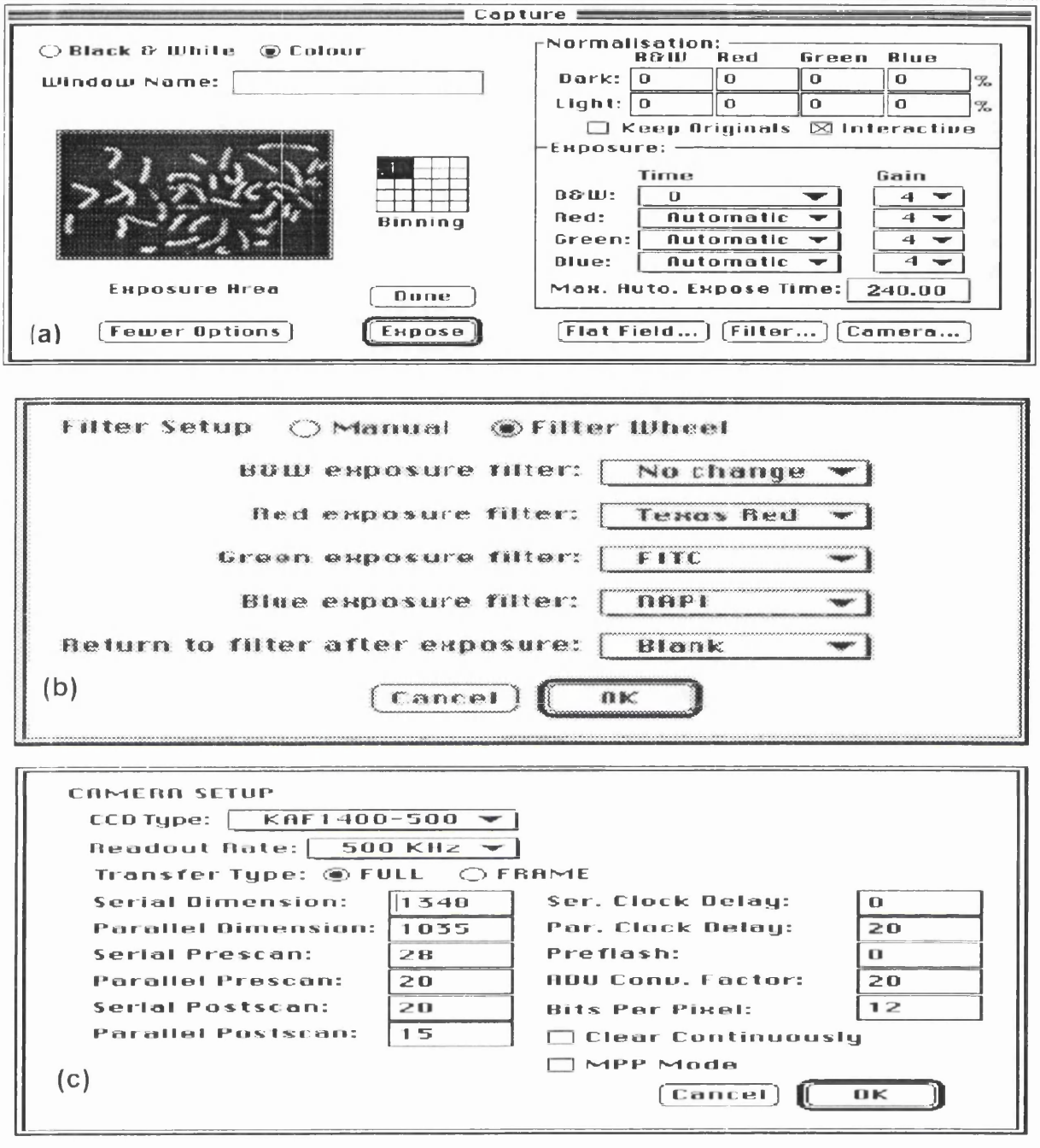


Figure 2. 2: CGH image capture settings. (a): Binning and exposure. (b): Filter set-up. (c) Camera set-up

Flat fielding

In some experiments flat fielding was performed to compensate for an image captured in non-uniform illumination. A blank image was first captured and used as an index to correct subsequent images taken by Capture. For a CGH image, a blank image was required for each colour component.

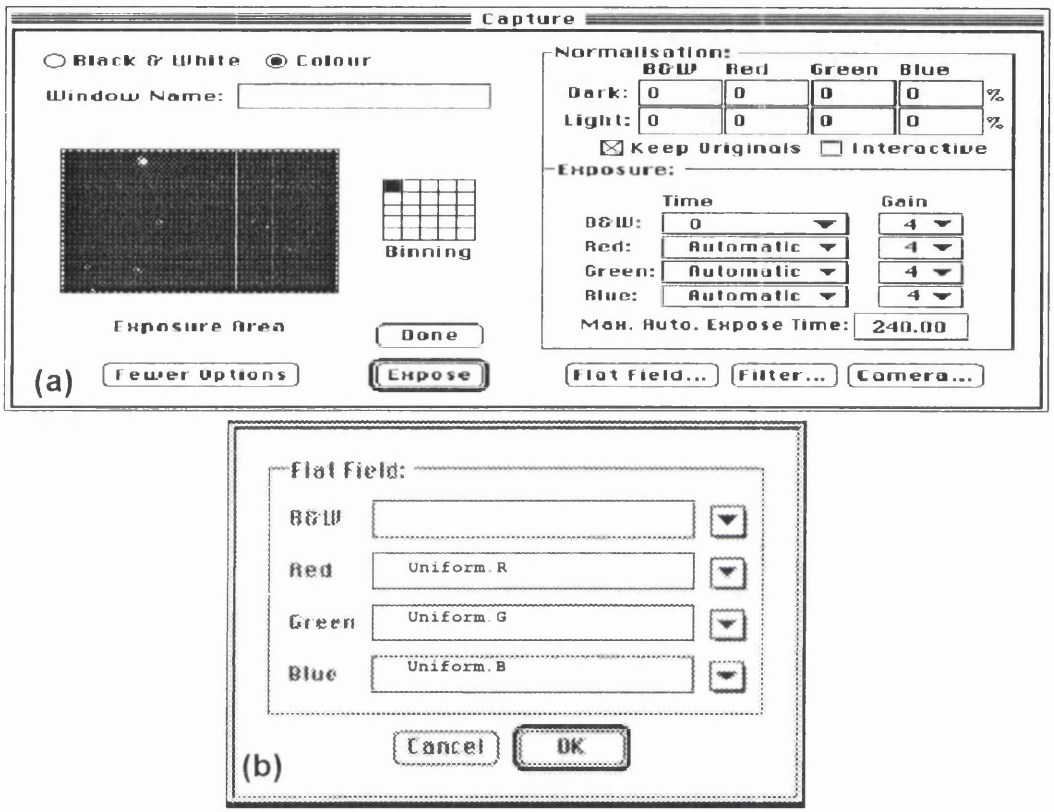


Figure 2. 3: Settings for flat fielding in the capture window. (a) Capture, exposure time, exposure gain and normalisation settings. (b) flat field dialog box

Using low power objective, a portion of the slide free of interphase nuclei and debris was selected. The focus adjustment was turned a fraction so that the surface was blurred. **Capture** was selected from the SmartCapture extensions menu and the settings displayed in Figure 2. 3 were selected. The blank image was captured and named as **Uniform.** In flat field window the Uniform.R, Uniform.G, and Uniform.B were selected for the red, green and blue windows respectively (Figure 2. 3.b).The metaphase of interest was then selected and captured as above. The captured image was corrected for

the light distribution measured at each point by comparing with the blank image.

Background removal

The function **BackgroundRemove** was used to remove the background from an image or to remove the effects of uneven illumination. The required image was opened and **BackgroundRemove** from the extensions menu was selected. **BackgroundRemove** settings were then chosen as depicted in Figure 2. 4.

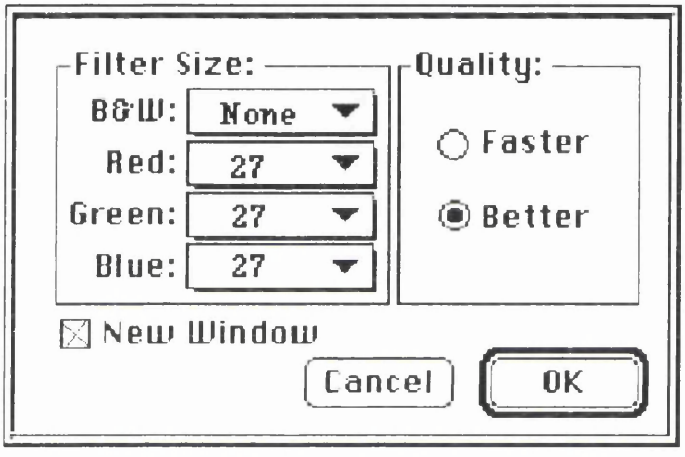


Figure 2. 4: BackgroundRemove window: Settings for CGH images

DAPI enhancement

The DAPI counter stain was enhanced to bring out the band structure and thus aid the identification of the chromosomes.

The required image was opened and **Split Colour Coord** from the Analyse menu was chosen. The blue image was selected and inverted to black and white by choosing **Adjust Contrast** from the Enhance menu and clicking on the reverse icon. After obtaining a satisfactory contrast by adjusting the slope of the contrast, the **Enhance** from the Ext. menu was called and the filter value was set to' Large-Moderate'. The resulting image was then used for chromosome identification based on its coarse banding pattern.

Thresholding

The segmentation script was run to define the chromosome boundaries by the DAPI counter stained image. The following settings were selected on the Segmentation window: 'binaries' box ' checked; 'green inside' box checked 'Show ROI histogram' checked. Segmentation script created a 'mask' which was then used to retain all material within the green area, setting the rest of the image to zero (Figure 2. 5).

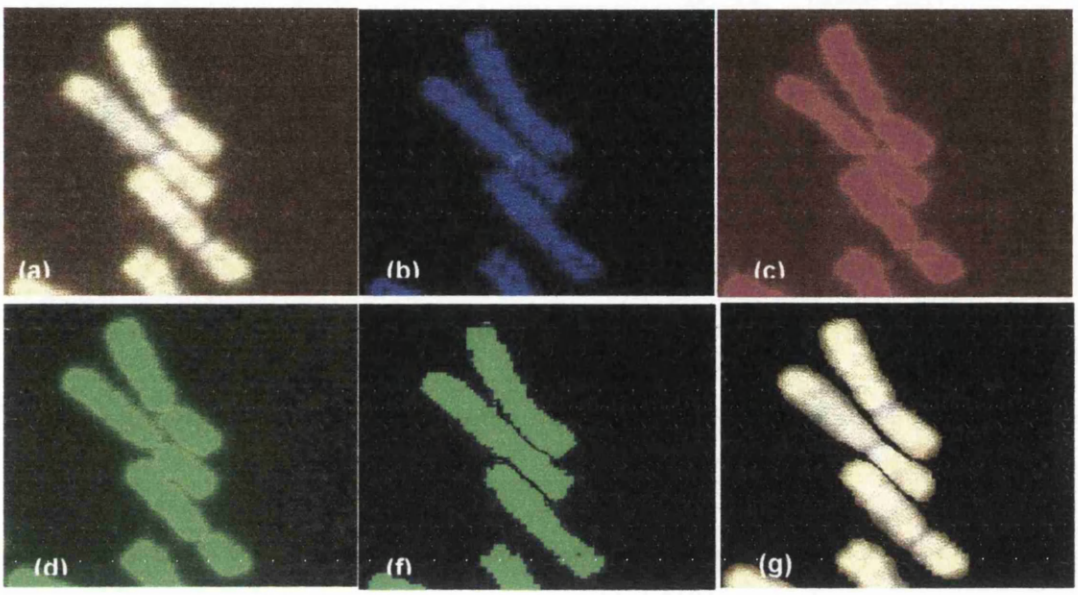


Figure 2. 5: Segmentation of the chromosomes in a CGH image. (a) CGH image before segmentation. (b-d) blue, red and green images. (f) masked chromosomes. (g) image with the 'cut out' chromosomes and zero background.

Fluorescence ratio (FR) equalisation

FR equalisation was carried out by calculating the average for each image and setting it arithmetically to 1. The FR equalisation operation was coded in a SmartCapture script (Equalisation).

Global analysis

The **ColourRatio** extension of the SmartCapture was used to map between ratios of red and green to identifiable colours.

The processed CGH image was opened before running the **ColourRatio** from the Ext. menu. The ColourRatio settings were set as shown in Figure 2. 6.

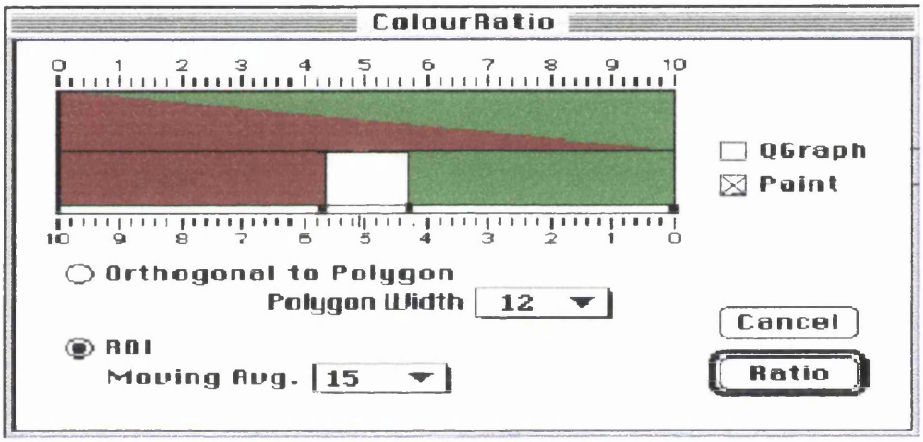


Figure 2. 6: ColourRatio window settings for CGH

The window was set to paint the image such that every pixel with a green/red ratio greater than 1 was green. If there was an excess of red fluorescence in any pixel it would be painted red. The **Ratio** button was clicked and after a few moments a new image appeared with the normal chromosomes painted white and both homologues of gained or deleted segments painted green or red respectively. These setting values were slightly different in each experiment. Widening the white gap in the ColourRatio window increased the specificity with loss of sensitivity. In mosaicism where less than 100% of cells

were abnormal, the white gap was narrowed to compensate the dilution of the abnormal cells.

Axial analysis

In this method, the orthogonally integrated FR distribution along the length of an individual chromosome was considered, a FR graph, and a chromosome image with the aberration marked in colour was generated.

Using the DAPI image to aid identification, the required chromosome was chosen. 'Polygon tool' from the status window was selected and the chromosome axis was marked along the length of the chromosome. The ColourRatio was selected from the Ext. menu. 'Qgraph' and the 'Paint' boxes were checked. The Ratio button was clicked resulting in a ratio coloured chromosome and a graph showing red/green ratio along the chromosome.

2.6.7 Quips CGH analysis

The Quips CGH analysis software is a suite of programs, that provide comprehensive patient management and semi-automatic image analysis for CGH studies. Each program in the suite may be used independently. The CGH/Karyotyper program provides for multiple metaphase image analysis to generate a single karyotype. The software suit and outline of the analysis are described in Table 2. 2 and Figure 2. 7.

Table 2. 2: Quips CGH Analysis Suite Programs (from Quips user's guide)

Program	*Description*
Quips DB	Record patient information and print text-only reports.
	Start other programs from the database to collect all analysis files into one folder for a case study.
SmartCapture	Capture a metaphase image using SmartCapture.
	Works with the SenSys KAF1400 camera to capture multicolored or monochrome images.
Quips CGH/Karyotyper	Edit and analyze a metaphase image to generate a karyotype and CGH ratio profiles.
	Provide access to a report editor for generating printed reports with images.
Quips Interpreter	Edit and analyze ratio profile data from several metaphases in a CGH experiment.

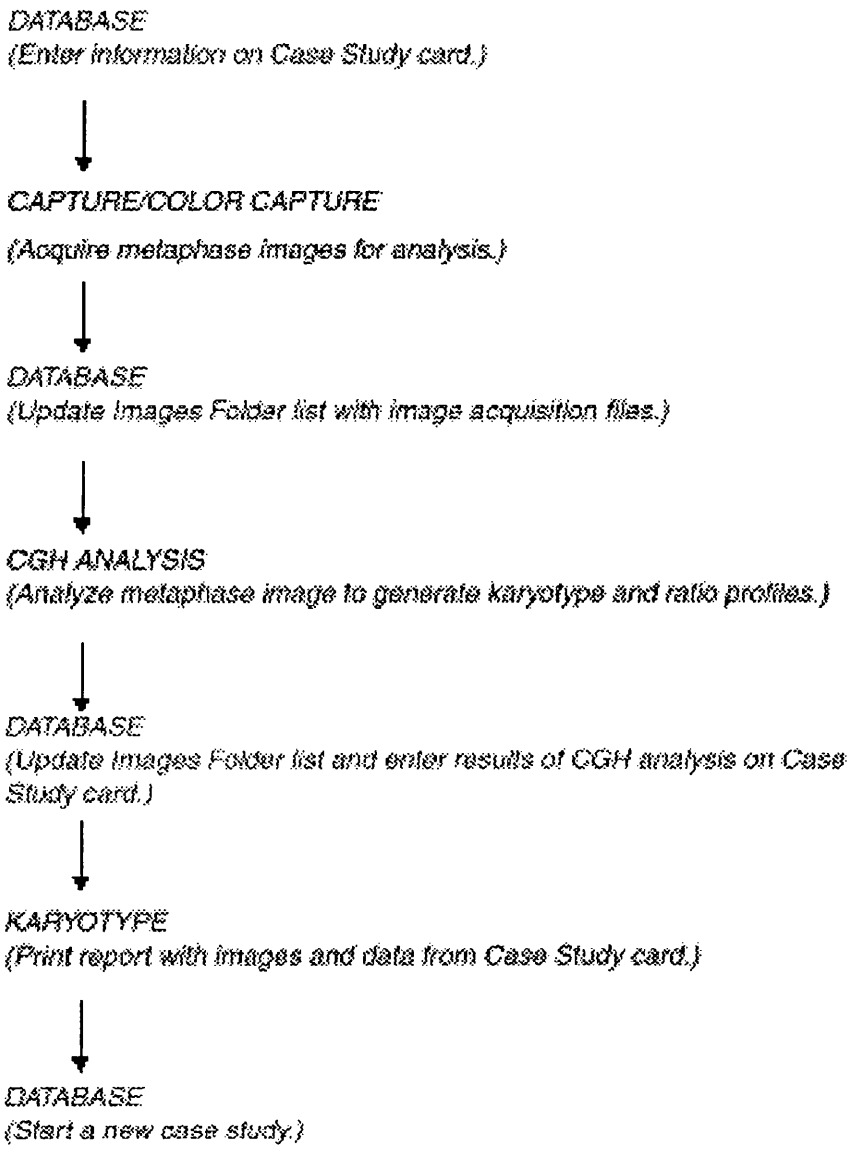


Figure 2. 7: Outline of CGH study using Quips CGH analysis software (from Quips user's guide).

2.7 The polymerase chain reaction (PCR)

Standard and Degenerate Oligonucleotide Primer PCR (DOP-PCR) were utilised during the various parts of this study. Standard PCR was mainly used as a tool to amplify DNA sequences which then were used as hybridisation targets in high resolution CGH. DOP-PCR was utilised to amplify whole genomic DNA for use as test samples when the original DNA sample was small.

2.7.1 Standard PCR

Solutions and reagents in a 50 μl PCR reaction tube:

Genomic DNA

0.1 μg

DNA polymerase (Amplitaq®)

1. unit

Reaction buffer (Perkin Elmer)

1 x

dNTPs:

dATP (200 μM)

dCTP (200 μM)

dGTP (200 μM)

dTTP (200 μM)

Primers: (forward and reverse)

30 pM each

SRY.PCR1: 5' GAA TAT TCC CGC TCT CCG GA

SRY.PCR2: 5' GCT GGT GCT CCA TTC TTG AG

DMD-1527 5' TTG AAT TAC ATT GTT TAA ATC CCA ACA TG

DMD 2170 5' CCT GAA TAA AGT CTT CCT TAC CAC AC

MSH2.PCR1 5' GAT TGT TAC CGA CTC TAT CA G

MSH2.PCR2 5' AAA ATG GGT TGC AAA CAT GC

XIST 1 5' GAA GTC TCA AGG CTT GAG TTA GAA G

XIST 2 5' ACA TTT TTC TCT AGA GAG CCT GGC

$MgCl_2$:

1.5 mM

Distilled water

Up to 50 μl

Procedure:

Depending on the purpose of study, PCRs were performed in a total volume of 100, 50 or 25 µl. Where necessary the concentration of each of the materials was optimised to get the best PCR result. Buffer, primers and dNTPs were added into pre-labelled 0.5 ml microfuge tube on ice then the volume was made up to the desired volume with distilled water. The mixture was subjected to 10 minutes of UV radiation before DNA and enzyme were added. The mixture was vortexed, centrifuged and overlaid with 50 µl of mineral oil before being transferred to an automated temperature-cycling machine (PCR machine). PCR reactions were performed as described in Table 2.3. PCR products were visualised by agarose gel electrophoresis (in 1 x TBE); ethidium bromide staining and transillumination on a UV-lamp as is described in section 2.7.5. Permanent records of these gels were made using a gel documentation system (UVP Imagestor 5000) linked to a thermal printer.

Table 2.3: Standard PCR

1 cycle:		
	94°C	2 minutes (initial denaturing)
30-40 cycles:		
	94°C	30-60 seconds (denaturation)
	53-60°C	30-60 seconds (primer annealing)
	72°C	1-3 minutes (primer extension)
1 cycle:		
	72°C	10 minutes (final extension)

2.7.2 DOP-PCR

Solutions and primer for DOP PCR:

10x reaction mix

20 mM $MgCl_2$,

500 mM KCl,

100 mM Tris HCl (pH 8.4)

1mg/ml gelatine

10x nucleotide mixture:

2 mM each of dNTP (dATP, dCTP, dGTP, dTTP)

10x DOP primer:

5'CCG ACT CGA AGN NNN NNA TGT GG3'

(N=A,C,G, or T)(Telenius et al 1992)

DNA polymerase

Amplitaq® (Perkin Elmer)

Distilled water

Procedure:

1 ng genomic DNA was used for DOP-PCR experiments. PCRs were performed in a total volume of 50 µl PCR reaction mix containing 1 unit of AmpliTaq® DNA polymerase; 5 µl 10x DOP-PCR buffer; 5 µl 10x nucleotide mixture, 5 µl 10x DOP primer and adjusted to 50 µl with H_2O. Reaction solutions without DNA or enzyme were used as negative controls. The reaction mixture was overlaid with 50 µl of mineral oil and transferred to an automated temperature-cycling machine (PCR machine). The optimised PCR program is described in Table 2. 4. The reaction products were then purified by ethanol precipitation or by using Millipore's Ultrafree ® -MC filter and controlled by gel electrophoresis.

Table 2. 4: DOP PCR profiles

1 cycle:		
	94 °C	2 minutes (initial denaturing)
5 cycles:		
	94°C	1 minute
	30°C	1.5 minute
	30-72 transition	3 minutes
	72°C	3 minutes
35 cycles:		
	94°C	1 minute
	62°C	1 minute
	72°C	3 minutes (with an addition of 1 sec/cycle)
1 cycle:		
	72°C	10 minutes (final extension)

2.7.3 Quantitative PCR

In this study, quantitative PCR was performed to confirm the CGH results in a case with abnormal Xq by quantifying the *XIST* locus copy numbers. Genomic DNA samples from a normal male, a normal female and the patient were subjected to series of optimised quantitative PCR experiments. The optimised quantitative PCR included 1 minute initial denaturation of the samples at 94°C followed by 20 cycles at 94^0C for 1 minute (denaturation), 55^0C for 1minute (primer annealing), and 72^0C for 1 minutes (primer-extension) and a final extension of 10 minutes at 72^0C, for one cycle. The PCR products were then visualised using a standard UV transilluminator and photographed using a gel documentation system (UVP Imagestor 5000) linked to a thermal printer. The photographs were then digitally saved as grey scale images in Macintosh PICT format. Using SmartCapture package the total values of signal intensities in one selected area of each band corresponding to patient's sample was calculated and compared to those from equal areas of bands from normal male and female samples (see Results).

2.7.4 Purification of PCR products

At the end of PCR, the amplified DNA was purified by removing the excess primers, dNTPs, and salts either by Millipore's Ultrafree ® -MC filter or by isopropanol precipitation.

Isopropanol precipitation of PCR products

Solutions and reagents:

Isopropyl alchohol
100%

Ethanol
70%

Ammonium acetate
4 M

TE buffer
10 mM Tris/HCl pH 7.0
1 mM EDTA

Procedure:

Mineral oil was gently removed from the top of the PCR product by a micropipette. Then one volume of 4 M ammonium acetate and two volumes of isopropanol were added, the mixture was vortexed and left on ice for 10 minutes, followed by 10 minutes centrifugation at 14000 rpm. The supernatant was discarded and the DNA pellet was washed in 70% ethanol, air dried and dissolved in appropriate amount of T.E. buffer pH 7.0.

Millipore Ultrafree ® filter units

The total reaction product was transferred to the upper part of the filter unit. The reaction vial was then rinsed with100 μl of 2 x SSC buffer which then was added to the filter unit. The filter unit was spun at 12000 rpm until all of the liquid had collected in the bottom of the microtube portion of the unit. This took about 12-15 minutes for a 150 μl sample. After that, another 100 μl of 2 x SSC was added to the filter unit which was centrifuged again. An aliquot

(same volume as initial total volume) of 1 x TE buffer (1 mM Tris-1 mM EDTA pH 7.6) was pipetted into the upper part of the insert to recover the purified DNA. This solution was transferred to a clean microcentrifuge tube. The whole filter unit assembly was discarded after use.

2.7.5 Identification of PCR products

Solutions, reagents and materials

Loading mix

0.25% bromophenol blue

0.25% xylene cyanol

40% sucrose

Agarose gel

1% (0.5g/50ml)

TBE buffer (pH 8.3)

10.8 g Tris

5.5 g boric acid

0.93 g EDTA

up to 500ml distilled water

Ethidium bromide

Procedure:

A 1% agarose gel was prepared by adding 0.5g agarose powder (SEA KEM GTG, FMC Bioproduct) to 50 ml 1 x TBE buffer. The mixture was then boiled in a microwave oven and allowed to cool down to 55°C before ethidium bromide (5 μg/ml) was added. The gel was then poured on to a casting tray. 6 μl of PCR product then was mixed with 1 μl of loading mix and loaded on the 1% agarose gel. The sample was then subjected to electrophoresis at constant 100 volts for 30 minutes. The DNA was then visualised using a standard UV transilluminator and photographed using a gel documentation system (UVP Imagestor 5000) linked to a thermal printer.

2.8 Plasmid/Cosmid Probe Culture and isolation

Solutions, reagents and materials

Luria-Bertani (LB) medium

Bacto-tryptone	5.0 g
Bacto-yeast extract	2.5 g
NaCl	5.0 g
Distilled water up to	500 ml

Sterilised by autoclaving

Starter culture or glycerol stock

American Type Culture Collection

CIRCLEPREP® Kit

Pre-lysis buffer

Alkaline lysis reagent

Neutralising solution

Isopropanol

100%

Ethanol

70%

Ammonium acetate

4 M

TE buffer

10 mM Tris/HCl pH 7.0

1 mM EDTA

Procedure

100 ml LB broth was inoculated in a sterile bottle with either 100 µl starter culture or 200 µl glycerol stock and incubated overnight (8-16 hours) at 37°C, in a shaking incubator. A Circleprep Kit was used to isolate the cosmid or plasmid DNA. The cells from overnight culture were spun down in 50 ml centrifuge tubes. Supernatant was then discarded and tube was drained for a minute. The cell pellet was resuspended by adding 4 ml of pre-lysis buffer before 4 ml of alkaline lysis reagent was added to lyse cells. After mixing the lysate 4 ml of neutralising solution was added and mixed before the tubes were spun at 12000 for 5 minutes at 4°C. Supernatant then was transferred to a clean 50 ml centrifuge tube and plasmid or cosmid DNA was precipitated

as described above. DNA pellet was then dissolved in TE buffer or distilled water.

2.9 Preparation of single stranded DNA by magnetic separation

Solutions, reagents and materials

Dynabeads-M-280 Streptavidin®
10 mg/ml
Magnetic Particle Concentrator
Dynal MPC®
Forward and reverse PCR primer
(one primer biotinylated)
Binding and Washing (B&W) buffer (2 x)
10 mM Tris-HCl pH 7.5
1.0 mM EDTA
2 M NaCl
Melting solution
0.15 M NaOH
DNA precipitation reagents
Ammonium acetate (5 M, pH 6.6)
Isopropanol
Glycogen (nucleic acid free)
Sterile water

Procedure

Dynabeads-M-280-streptavidin was used to prepare single stranded DNA from PCR products, which were then used as targets in hybridisation studies. Biotin was introduced into one of the strands of the DNA during the PCR amplification using one of the primers biotinylated in the 5' end. PCR amplifications of DNA targets were performed as described above. The PCR product was then immobilised through the interaction between biotin and Streptavidin onto magnetic beads with covalently coupled Streptavidin on the surface. 5-20 μl of Dynabeads M-280 Streptavidin was washed with 20 μl of 2 x B&W and resuspended in 40 μl of 2 x B&W buffer. 40 μl of the amplified PCR product was added to 40 μl of the pre-washed Dynabeads and incubated for 15 minutes at room temperature to immobilise the biotinylated PCR product. The Dynabeads were kept suspended by gently tapping the

tube. The tube containing the immobilised product was placed in a suitable magnet (Dynal MPC) and the supernatant was removed with a pipette.

The magnetic beads with the immobilised DNA, collected on the side of the tube were washed by adding 40 μl of 2 x B&W buffer to remove the reaction components. The washed, immobilised product was placed in the Dynal MPC and the supernatant was removed.

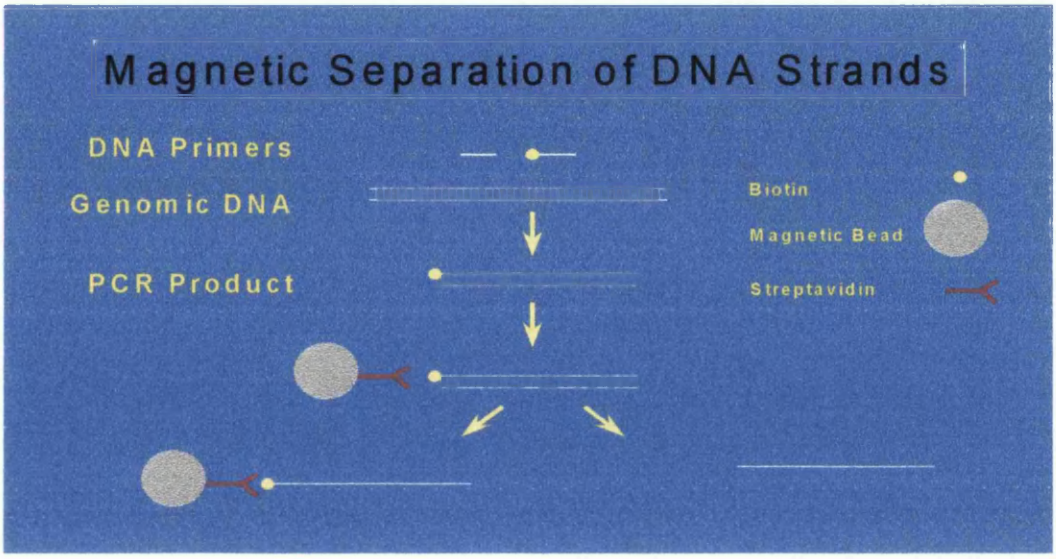


Figure 2. 8: Schematic diagram for magnetic separation of DNA strands. Biotin is introduced into one of the strands of the DNA during the PCR amplification using one of the primers biotinylated in the 5' end. The PCR product is then immobilised through the interaction between the biotin and Streptavidin covalently coupled on the surface of magnetic bead. After immobilisation, the magnetic beads are washed to remove the reaction components. The immobilised double stranded DNA is converted to single stranded template by alkaline denaturation and subsequent removal of the biotinylated strand by magnetic separation.

The immobilised double stranded DNA was converted to single stranded template by alkaline denaturation and subsequent magnetic separation. The non-biotinylated ssDNA was eluted from pelleted beads by the addition of 300 μl 0.15 M NaOH for 5 minutes. After magnetic separation, the supernatant was transferred to a new tube and precipitated by neutralising with the addition of 150 μl 5 M ammonium acetate pH 6.6, followed by isopropanol precipitation using 2 pg of nucleic acid free glycogen as a co-

precipitant as described earlier. Finally DNA pellet was dissolved in an appropriate buffer.

2.10 DNA molecules as hybridisation targets

In the final part of this study, the feasibility of using DNA molecules instead of metaphase chromosomes as hybridisation targets to improve the resolution of the CGH technique was investigated. Combed fibres or high concentration spots of cosmid DNA containing 35 kb of human X chromosome attached to glass slides were targeted in comparative hybridisation studies. Spots of DNA arrays corresponding to *DMD*, *SRY* or *hMSH2* genes amplified by PCR and attached to glass slides were the subjects of CGH experiments.

2.10.1 Slide preparation

Slides used as solid base for DNA molecules had been either silanised or pre-treated with Poly-l-lysine. All procedures were carried out at room temperature and with double distilled water unless otherwise stated.

Silanisation of glass supports

Method 1

Solutions and reagents

HCl
Methanol
H_2SO_4
3-aminopropyltriethoxy silane (Sigma)
Ethanol

Procedure:

Slides were cleaned in equal parts of concentrated hydrochloric acid and methanol for at least 30 minutes followed by overnight immersion in 18 M sulphuric acid. The slides were then washed in 8-10 changes of ultra-pure water, immersed in boiling water for 10 minutes and air dried. Cleaned slides were placed in a 10% solution of 3-aminopropyltriethoxy silane (Sigma) in 95% ethanol for 1 hour, rinsed several times with water, washed with ethanol

and air dried. Prior to use, slides were heated to 80°C overnight (Weier et al 1995).

Method 2

Solutions and reagents

Aminophenolpropyl silane (Sigma)
Methanol
Acetone
Ethanol

Procedure:

Glass slides were immersed in a 2% aminophenolpropyl silane in acetone for 40 minutes at room temperature followed by three 5-minute washes in 50% acetone (Joos et al 1997). Prior to use, slides were heated to 80°C overnight.

Poly-l-lysine treatment

Materials:

NaOH
Poly-l-lysine solution
70 ml Poly-l-lysin
280 ml Distilled water
Ethanol

Procedure:

50 g of NaOH pellets were dissolved into 150 ml distilled water before 200 ml of 95% EtOH was added. If solution remained cloudy, distilled water was added until clear. Thirty slides were immersed in the solution for 2 hours and then rinsed 3 times with distilled water. The slides were then transferred to lysine solution and left to soak for 1 hour. Excess liquid was removed and the slides were dried at 40°C for 5 minutes and stored in a closed box for at least two weeks prior to use.

2.10.2 Comparative hybridisation to DNA fibres

Linearised cosmid fibres were attached to a glass surface and aligned in parallel by molecular combing. Dual colour FISH was performed on these cosmid fibres with probe mixtures containing different ratios of FITC and

TexasRed-labelled MA2B3 cosmid DNAs. To explore the feasibility of improving the CGH resolution, metaphase chromosomes were replaced by arrays of DNA fibres spotted on glass slides. The DNA spots were then the subjects of a series of CGH experiments.

Preparation of probe and target DNA

Cosmid MA2B3 was employed both as probe and as combed target DNA in this part of the study. It contains about 35 kb of the human dystrophin gene, including exon 48 (Tocharoentanaphol et al 1994).

Linearisation and staining of the cosmid DNA

Solutions, reagents and materials

Cosmid MA2B3

***Not I* enzyme**

GIBCO-BRL

Reaction buffer

50 mM Tris-HCl (pH 8.0)

10 mM $MgCl_2$

I00 mM, NaCl

YOYO-1 dye

Molecular Probes

MES buffer

[2-(N-morpholino) ethanesulfonic acid; (Sigma), pH 5.5].

Procedure

The MA2B3 cosmid was linearised with *Not* I. One unit of the *Not* I was required to cleave 1 μg of cosmid DNA in one hour at 37°C in the reaction buffer. *Not* I digestion resulted in linearisation of the cosmid with the vector at one end of the fibre. In some experiments the linearised DNA was stained in a 5:1 ratio [base pairs per dye (YOYO-1)] immediately before use as controls. YOYO-1 stained cosmid DNA was diluted to 1 μg/ml in 50 mM MES buffer.

Alignment of DNA by a moving interface (Molecular combing)

A drop of stained or un-stained DNA solution (typically 5 μl) was deposited on a coated coverslip or slide. An untreated coverslip was then floated on

top, forcing the drop to spread to a final thickness of ~20 μm. After a few minutes the coverslip was carefully removed with forceps in such a way that a continuous and directed receding air-water meniscus was produced, resulting in a parallel alignment of cosmid fibres. The density and the alignment of YOYO-1 stained DNA fibres on the coverslips were monitored by microscopy (Bensimon et al1994).

Dual-colour FISH to aligned DNA fibres

Pre-hybridisation and hybridisation

Materials, reagents, and solutions

Labelled cosmid DNA

MA2B3 FITC or TexasRed labelled

Competitor DNA

Cot-I DNA (BRL Life Sciences)

Cold ethanol

70%, 90%,100%

Deionised formamide

Hybridisation solution

50% formamide, 10% dextran sulphate (Pharmacia)/ 1 x SSC

1 x SSC.

Denaturation solution:

70% formamide, 1 x SSC pH 7.0

Rubber cement

Procedure:

Slides were baked at 60°C overnight. After 5 minutes wash in 2 x SSC, slides were put through an alcohol series of 70%. 90% and 100%. and air-dried. Preparations for immediate use were kept at room temperature and used within one week for comparative hybridisation experiments. Aligned DNA fibres were denatured for 2 minutes at 70°C in 70% formamide, 1 x SSC, pH 7.0. The DNA fibres then were dehydrated through an alcohol series. For comparative hybridisation experiments cosmid MA2B3 DNA was labelled with either FITC or TexasRed by nick-translation as described above. The

chosen ratios of FITC to TR labelled DNA fragments in the hybridisation solution were 1:2, 2:3, 1:1, 3:2, 2:1 and 3:1. Ten microlitres of hybridisation solution contained between 22.5 and 90 ng of each fluorochrome-labelled cosmid DNA with a fragment length of 500-3000 bp and 5 μg of human Cot-I DNA (BRL). After denaturation at 75°C for 10 minutes the hybridisation solution was applied to the slide with the denatured, aligned DNA fibres covered by an 18x18 mm coverslip and sealed with rubber cement. Hybridisation took place at 37°C overnight.

Post-hybridisation

Washing steps

Solutions, materials and reagents

1 x SSC.	1.5 M NaCl, 15 mM Na_3-Citrate
2 x SSC	
1 x ST solution:	4x SSC, 0.05% Tween-20
Washing solution A	50% formamide, 2 x SSC
Alcohol series	70%, 90%,100% ethanol
Coplin jars	

Procedure:

Washing for FITC or TR labelled DNA fragments was carried out as described above for standard FISH. Slides were then air-dried and mounted in antifade with no counterstain.

Image analysis

Aligned DNA fibres subjected to comparative hybridisation were visualised and evaluated using basically the same system and approach as used for CGH and FISH.

Each fluorochrome was imaged separately using the SmartCapture software. Optimal exposure times and optical settings were established empirically and then kept constant for the entire set of DNA fibres recorded for a given experiment. Each fibre was segmented and the total fluorescence intensity in the entire segment for each fluorochrome was measured. Then to estimate

the background, a neighbouring area with the same shape and size was selected and the value for green and red colours was calculated. Corrected fluorescence value for each fibre was calculated by subtracting the total background fluorescence value from the total fluorescence value obtained along the target fibre. The fluorescence ratio of each fibre was obtained by dividing the corrected green by the corrected red fluorescence intensity.

CGH to dotted DNA fibres

Dotting of DNA on glass slides

Essential materials

Linearised cosmid DNA
Silanised or poly-l-lysine coated glass slides
Glass capillaries

Procedure

Linearised cosmid DNA was dotted onto silanised or poly-l-lysine coated glass slides at a concentration of 1 μg/ μl using a micropipette or glass capillaries under an inverted microscope. Glass capillaries with a size of 1.2 mm outer diameter and 0.68 mm inner diameter were mounted on a micropipette puller (Sutter Instruments P-87). By applying heat in the middle of the capillary and pulling forces at the ends two needles with open ends were produced. For each experiment two hybridisation areas were produced on each slide. For each hybridisation area, 16 DNA spots were spaced at a distance of 4-10 μm (where glass capillaries used under the inverted microscope) to 400 μm (where micropipettes used) from centre to centre (see Results).

Pre-hybridisation and hybridisation

Slides containing dotted DNA were aged for 1 hour in an oven at 80°C before being dehydrated in an ethanol series (70%, 90%, and 95%). Hybridisation was carried out at 37°C for 48 hours. Washing steps were performed in

exactly the same manner as standard CGH. Hybridising DNAs were derived from normal male and normal female individuals.

Each hybridisation area was the subject of a CGH experiment with similar test and control DNAs but with different colours. For instance if the test and control DNAs respectively had been labelled with FITC and TexasRed in one hybridisation area, it was the opposite for the other area. This measure was made to detect and deduce the effect of backgrounds corresponding to each fluorochrome.

Image analysis

DNA fibres subjected to CGH were visualised and evaluated using basically the same system and approach as used for single DNA fibres. Each fluorochrome was imaged separately using the SmartCapture software. Optimal exposure times and optical settings were established empirically and then kept constant for the entire set of DNA spots recorded for a given experiment. All spots in a single microscopic field were segmented and the total fluorescence intensity in the entire segment was measured for each fluorochrome. Fluorescence values corresponding to test or control DNA in one hybridisation area were compared to those from the same sample in another area with different colour. Using a statistical analysis package (Minitab release 10.5) a regression analysis was performed and the background values for each colour were calculated. Finally, the corrected fluorescence value for each spot was calculated by subtracting the background fluorescence value from the total fluorescence value obtained from the target spot and the fluorescence ratio of each spot was obtained by dividing the corrected green with the corrected red fluorescence intensity.

2.10.3 CGH to DNA microarrays

Hybridisation targets

Three target sequences, corresponding to the exon 48 of human *DMD* gene on chromosome X, *SRY* gene on chromosome Y and *hMSH2* gene on chromosome 2 were selected and amplified by PCR using genomic DNA

templates. The PCR products were made single stranded using magnetic beads (2.9). The single stranded DNA products then were spotted on silanised glass sequentially in a manner that the DNA targets corresponding to the X and Y chromosomes were separated by a spot corresponding to chromosome 3 DNA. In each slide, 16 spots were produced (Figure 3. 40).

Pre-hybridisation and hybridisation

Pre-hybridisation and hybridisation steps were the same as described for CGH to DNA fibres. DNA samples from normal male and normal female individuals were used as test and control DNAs in CGH experiments.

Image analysis

For image analysis, captured images from spots corresponding to *DMD*, *SRY* and *hMSH2* were taken as one image. Each fluorochrome was imaged separately using the SmartCapture software. Optimal exposure times and optical settings were established empirically and then kept constant for the entire set of DNA spots recorded for a given experiment. All spots in a single image were segmented and the total fluorescence intensities for each fluorochrome in the entire segment of each spot was measured. The ratios of averaged values of red and green signals corresponding to the autosome spots were calculated and were used as correction values for the normalisation of averaged red and green fluorescence values corresponding to *DMD* ands *SRY* spots. Finally the corrected fluorescence value for each spot was calculated and the fluorescence ratio of each spot was obtained by dividing the corrected green with the corrected red fluorescence intensity.

3:Results

3.1 Optimisation of the CGH technique for clinical cytogenetics laboratory

3.1.1 CGH using published protocols

Ten samples, 5 from normal individuals and 5 from patients with known abnormalities were used to compare the main published approaches to CGH (Table 3. 1). The first protocol (method 1) reported by Kallioniemi et al (1992) used directly labelled test and control DNAs for hybridisation while the second (method 2), published by du Manoir et al (1993), was based on an indirect labelling approach.

Table 3. 1: CGH using two main published approaches, summary of results

Sample	Patient's karyotype	Control DNA	Method 1		Method 2	
			Image quality*	Result	Image quality*	Result
1	46,XX	46,XY	1/5	+X,-Y	1/5	+X,-Y
2	47,XX,+8	46,XY	-2/5	FAIL	-1/5	FAIL
3	46,XX	46,XY	1/5	+X,-Y	1/5	+X,-Y
4	46,XY	46,XX	1/5	+Y,-X	-1/5	FAIL
5	46,XX,+21	46,XY	-3/5	FAIL	-2/5	FAIL
6	46,XY	46,XX	0/5	+Y,-X	-1/5	+Y,-X
7	46,XX	46,XY	1/5	+X,-Y	3/5	+X,-Y
8	47,XXX	46,XX	2/5	+X	2/5	+X
9	47,XY,+13	46,XY	-3/5	FAIL	-1/5	FAIL
10	47,XXY	46,XY	1/5	+X	3/5	+X

* Image quality was scored between-5 to 5

Test and control DNA samples for each series of experiments were identical for both methods. To allow the two methods to be performed in similar conditions, target metaphases were also from the same batches of slides.

Although the intensity of signals was directly related to the copy numbers of test and control DNAs in both protocols, some difficulties were encountered with both methods. In the first approach while the hybridisation was uniform, the intensity of signals was weak causing difficulties in image capture and analysis in some cases. In the second approach, while the intensity of signals was acceptable, chromosomes were not uniformly hybridised. These difficulties caused problems in CGH analysis in samples from 7 patients resulting in the need to repeat the experiments (Table 3. 1-2). Therefore, some optimisation was necessary.

Table 3. 2: Comparative genomic hybridisation, using two main published approaches

	Kallioniemi et al (1992)	du Manoir et al (1993)
No.of samples	10	10
Hybridisation time	48-72	48
Labelling Method	Direct	Indirect
Hybridisation pattern	Uniform, faint signals	Uneven, strong signals
Post-hybridisation time	1-2 Hours	5-6 Hours
Failures	3	4
Reason(s) for failures	Weak signals	Uneven signals or high background

3.1.2 Modification of the CGH technique

Twenty DNA samples from patients with known chromosome abnormalities were selected for this stage of the study. Each sample was used as a test DNA and compared with a normal DNA of opposite sex. While investigating the effect of one parameter, all other parameters including the test and control DNAs were kept unchanged.

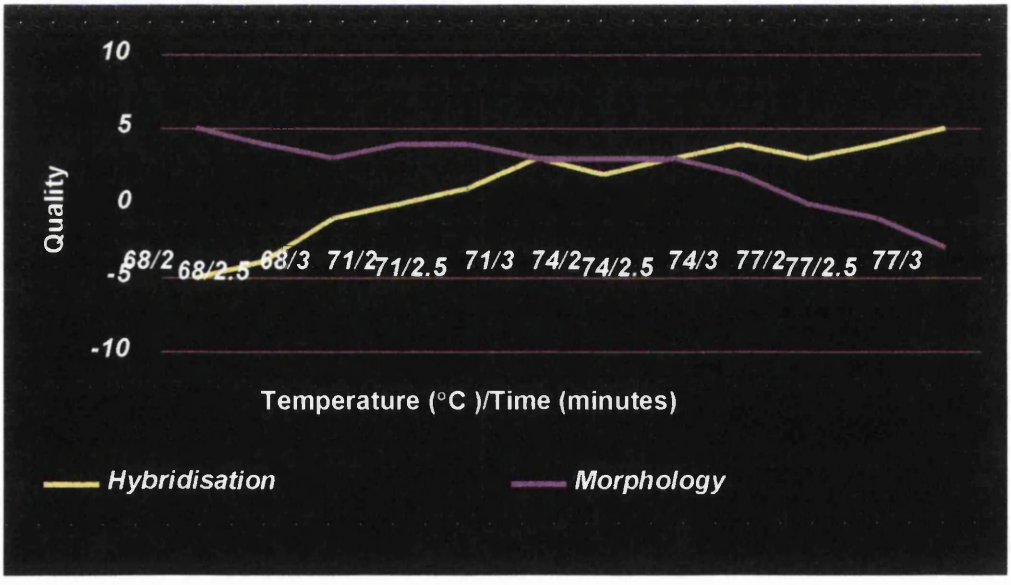
Target metaphase

Although metaphase preparations had passed the initial evaluation (see Methods) and appeared suitable for CGH, they did not necessarily produce optimal CGH results. Slides prepared from the same batch always produced the same quality of CGH images.

Denaturation temperature and time

In all slide batches, raising the denaturation temperature up to 80°C, and increasing the denaturation time up to 3 minutes caused an improvement in the hybridisation quality. These measures however, damaged the morphology of the chromosomes.

Extensive retrospective and prospective studies on slide batches which had already been shown to produce acceptable results showed that incubation for 3 minutes at 74°C produced optimum CGH results (Figure 3. 1).



* Quality was scored between-5 and 5

Figure 3. 1: Effects of denaturation time and temperature on target chromosome morphology and hybridisation quality.

DAPI morphology

Numerous CGH experiments showed a consistent direct relationship between the quality of CGH and DAPI images. The same concentration of DAPI counterstain solution produced clean, bright blue signals in previously proven successful slide batches but dirty and/or faint DAPI signals in those that had failed to produce acceptable CGH results. Thus, it appeared that the quality of the CGH experiment could be predicted by looking at the DAPI appearances of metaphase and interphase cells under 10x magnification even before checking the actual fluorochrome signals at 100x magnification.

Several acceptable and unacceptable slide batches were then subjected to denaturation experiments by immersing them in 70% formamide for 3 minutes at 74°C. The denatured preparations were dehydrated through an alcohol series before counterstaining with DAPI. The slides were then evaluated under a fluorescence microscope. Slides with proved quality consistently showed bright and clean blue signals while those that had proved to be unacceptable in CGH experiments produced faint and/or dirty DAPI signals (Figure 3. 2).

It was therefore concluded that the intensity of DAPI signal arising from interphase and metaphase chromosomes after denaturation was a reliable predictor of the usability of each slide batch for future CGH experiments.

The approach described here then used for future experiments to cut down on waste.

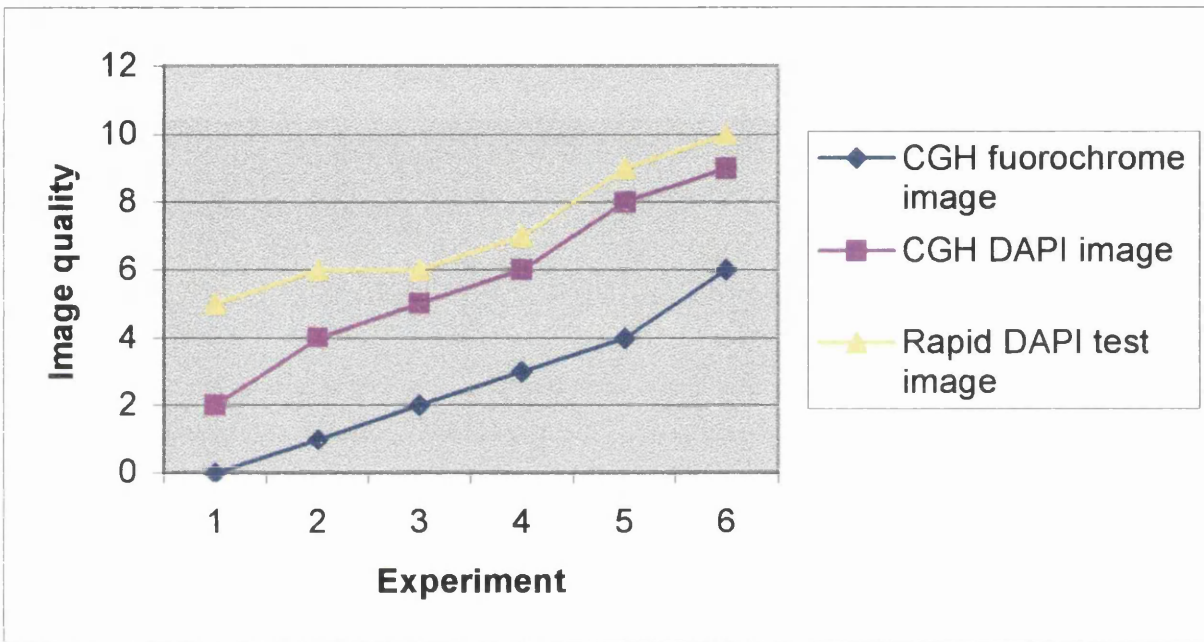


Figure 3. 2: Correlation between CGH fluorochrome quality, CGH DAPI image and rapid DAPI test image.

Slide ageing

Baking the slides after dropping the chromosome preparation had an adverse effect on the hybridisation quality. The longer slides were exposed to high temperature the worse the results became. Slides that had been dried on a hot plate resulted in the poorest CGH images. The best results were achieved when the slides were allowed to air dry and then aged at room temperature for 24 hours (Figure 3. 3). However, in urgent cases acceptable results were obtained by incubation of the slides for 1 hour at 60°C in a dry incubator (oven). It was also found that fresh slides gave better results. Use of slides older than one week resulted in poor CGH images. The best option for long term storage of slides was to store the slides (after alcohol dehydration) in boxes sealed with Parafilm ® in -70°C freezer. The quality of results however, was not comparable to those obtained from fresh slides.

Other parameters

Proteinase K treatment of the slides and several modifications of methanol/acetic acid (3/1) fixation caused no significant improvement in hybridisation quality.

Genomic DNA labelling

Various parameters in DNA labelling were investigated (Table 3. 3).

Table 3. 3: Factors investigated in genomic DNA labelling.

Nick translation time
Nick translation temperature
Direct or indirect labelling
Probe size

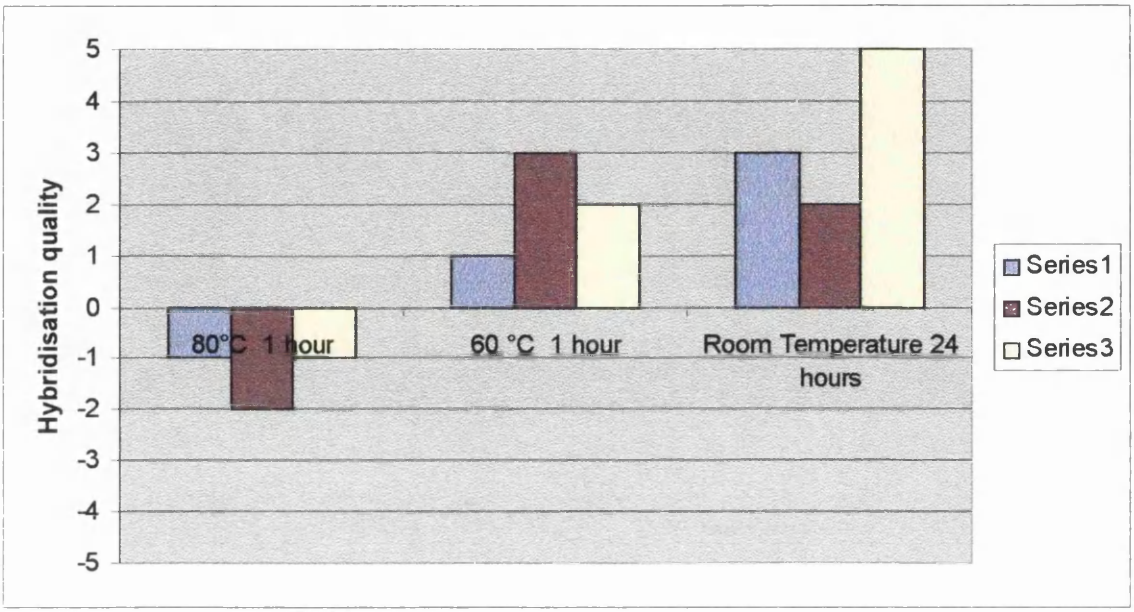


Figure 3. 3: Effects of the methods of slide ageing on the CGH image quality. Better results were usually obtained when slides had been stored at room temperature for 24 hours.

Direct or indirect method

For optimisation, test and control DNA probes for CGH were either labelled directly (with fluorochromes) by incorporating fluorochrome-coupled nucleotides, or indirectly, by incorporating reporter molecules (biotin or digoxigenin), which were then detected via fluorochrome-coupled reporter binding molecules. Directly fluorophorated genomic DNA resulted in smooth signals along the chromosomes, which is essential for assessing the fluorescence intensities at each point along the chromosomes. They could also be visualised immediately after the post-hybridisation washes. However, the signal strength obtained was only about 20 percent of that produced by an equivalent amount of labelled DNA detected by secondary reagents. This was problematic even though a sensitive digital imaging device was used for signal detection. The problem was overcome when the amount of the test and control DNA in the hybridisation solution was increased to 1 μg /hybridisation area (instead of 200 ng as described by Kallioniemi et al 1992-1994). The major drawback of the indirect method was high background, uneven hybridisation and complex and time consuming post-hybridisation steps. Manipulating various factors including Cot-I and DNA concentration did not give acceptable results. For these reasons, the modified direct method was adopted as the method of choice for DNA labelling during the course of this study.

Probe size

Probe sizes could be adjusted by manipulating several parameters in the nick translation reaction. Standard concentrations of DNase and other reagents were used.

Nick translation time and temperature were parameters which were manipulated for optimisation. Increasing the temperature (14-16°C) and the length of incubation time (30-180 minutes) resulted in decreasing the size of the probe. By changing the incubation time between 30-180 minutes the probe size ranged from mostly 200 bp to mostly >2000 bp.

CGH experiments performed using probes with sizes between 500-1200 bp resulted in the optimum results (Figure 3. 4). Those were the probes that had been obtained by a nick translation time and temperature of 55 minutes at 14°C.

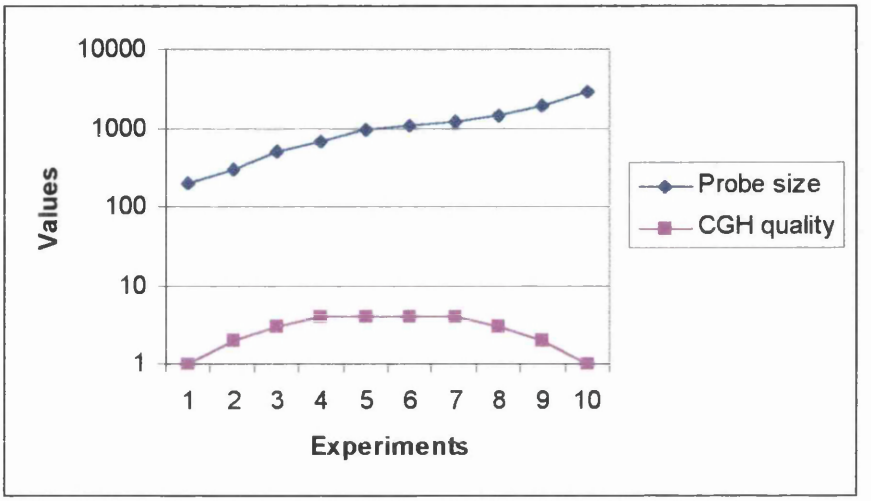


Figure 3. 4: Correlation between the CGH image quality and the probe size. Increasing the probe size between ~200 bp-1200 resulted in the improvement of CGH quality. Probes longer than 1200 bp resulted in increasingly lower quality CGH images.

In the early stages of the study, to validate the probe size, each sample was run on a 1 percent neutral agarose gel against known size standards. However, after the technique was optimised, this step was abandoned because the sizes of the probes were usually in the required range.

DNA hybridisation

Several modifications in the standard CGH technique ensured stronger hybridisation signals. Those modifications included higher amount of labelled test and control DNA and lower quantity of unlabelled human Cot-I DNA (20-30 μg instead of 50 μg in the hybridisation buffer) (Figure 3. 5). No significant differences between the quality of images in CGH experiments performed using 48-hour and 72-hour incubations were found. Therefore, in midweek and urgent experiments, 48-hour approach was adopted. However, in most circumstances, it was more convenient to follow the 72 hours approach by

performing the labelling and hybridisation on Fridays and the post-hybridisation steps and image analyses on the following Mondays.

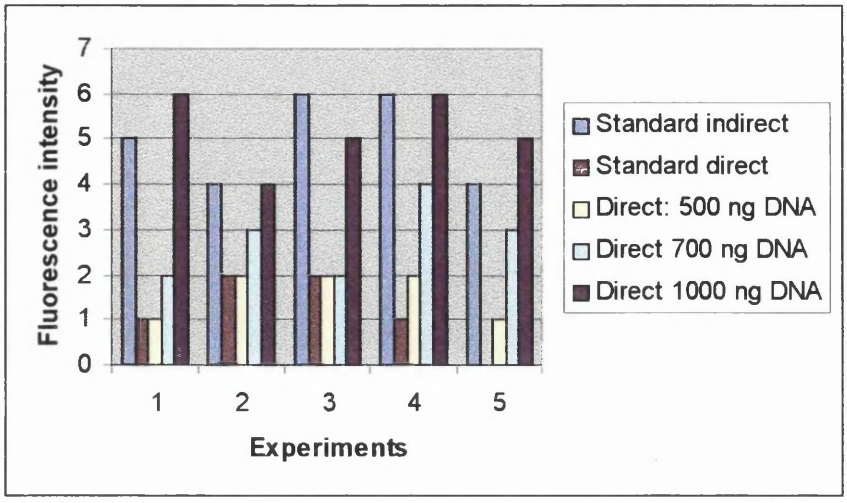


Figure 3. 5: Effects of alteration of the DNA concentration in hybridisation solution on the fluorescent intensity. Fluorescent intensity obtained by 1 μg of directly labelled DNA was similar to that obtained by standard indirect method.

Post-hybridisation washings

As the direct method had been adopted as the preferred approach for probe labelling, post-hybridisation detection of signals was not necessary. Therefore, only post-hybridisation washing steps were subject to optimisation procedures. Various current washing protocols were tested and a modified protocol which included 2 x 7.5 minutes washing of slides in 50% formamide in 2 x SSC, followed by 1 x 5minute in 1 x SSC, 1 x ST buffer at 42°C and 1 x 5minute in ST buffer at room temperature was developed. This protocol resulted in a very simple and fast method for post-hybridisation washing.

Image processing and analysis

During the optimisation of the lab-based parts of the CGH, image processing and analysis was carried out by standard approach using SmartCapture software (Digital Scientific, Cambridge, V2.1). That approach included flat

fielding, background removal, image enhancement, thresholding and fluorescence equalisation followed by global analysis, axial analysis, scoring, and the final analysis (see Methods). At least two working days were required to analyse 10 metaphases from each sample.

Several modifications made in the standard approach for CGH image capture and analysis simplified the whole CGH process and made it faster and more reliable.

Flat fielding

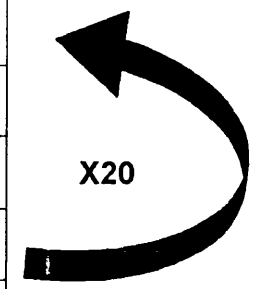
Significant uneven illumination was present only in early stages of this study where the quality of slides was poor. However, after various modifications in labelling and hybridisation stages, flat fielding was found to be unnecessary. On the other hand, when the signal quality was poor, even flat fielding failed to produce acceptable results.

Automatic image processing

First 20 images were captured and saved as 20 different files. Using a feature provided in SmartCapture, for each experiment one script was written to automate the very time consuming image processing parts of the CGH analysis. Each time up to 20 metaphases were processed while no supervision during the processing was necessary. For writing the scripts function (F) keys of the computer keyboard were defined sequentially so that writing each script took no more than maximum 5 minutes time.

Table 3. 4: Script used for processing of 20 images

Command	Function Key
Open file 1	F5
Remove background	F6
Save as 1*	F7
Segment chromosomes	F8
Equalise colour	F9



Rapid CGH analysis

Retrospective study of 11 cases with normal or abnormal CGH results showed that axis analysis and scoring had provided no further information in abnormal cases or unmasked new abnormalities in cases which had already appeared to be normal in global approach. In normal cases, global analyses of 5 high quality CGH images were found to be enough for ruling out unbalanced abnormalities, as analysis of more metaphases (up to 20) had given no more information.

Because of these findings, a new approach for rapid and reliable CGH image analysis was designed. Based on that approach, 5 high quality CGH metaphases were analysed by global analysis. Where no abnormality was detected the case was considered to be carrying no unbalanced abnormalities. If one or more abnormalities were suspected then up to 20 metaphases were analysed globally and estimated breakpoints and relative copy numbers were defined.

Using the approach described, in a blind study, nine cases were studied. All results were compatible with those obtained by conventional banding method. Additional axis image analysis together with scoring provided no further information

Detailed image analysis including axial analysis and scoring was only performed when precise definition of the breakpoints was required. Detailed CGH analysis was also performed to study the behaviour of the fluorescence in telomeric regions as described in 3.3.2.

3.2 Test of modified protocol using known cytogenetic abnormalities

Fifty-six samples from normal individuals or patients with constitutional chromosome abnormalities were the subjects of a blind study using the modified CGH protocol. Results were then compared with those obtained by conventional methods. CGH successfully detected all unbalanced abnormalities. The results are summarised in Table 3. 5 (see also Figures 3.6 and 3.7).

Table 3. 5: Test of modified protocol using known cytogenetic abnormalities, summary of results:

Phenotype/genotype	
Affected/abnormal cases	41
Normal/normal individuals	12
Normal/balanced	3
Types of unbalanced abnormalities	
Autosomal (whole chromosome)	21
Sex chromosome (whole chromosome)	13
p or q arm only (complete or partial)	6
Marker chromosomes	1
Sources of DNA samples	
Blood	5
Bone marrow	1
Fixed cells	26

Frozen cells	24
Failures:	
Failure to obtain result from the first CGH test	4
Failure to get result even by repeated experiments	0
Average experiment/sample required to achieve results	1.13
CGH/G-banding discrepancies	0
Time	**Day (s)**
Average time required for post-hybridisation steps and image analysis (max/min)	1.87 (4/1)

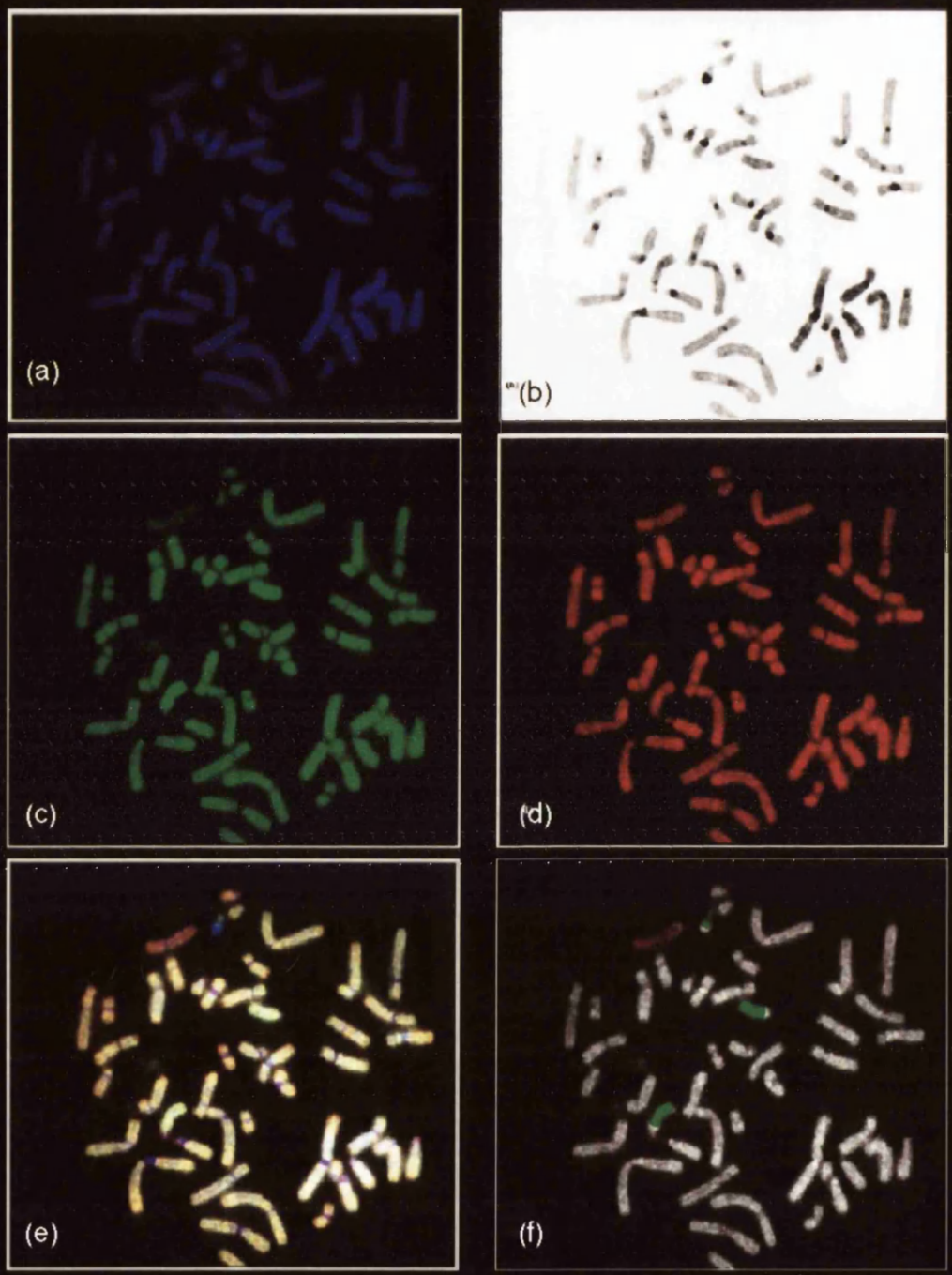


Figure 3.6: CGH global analysis of a case with trisomy 13. (a) CCD image of a normal metaphase spread counterstained with DAPI to allow chromosome identification. (b) Digital inversion of (a) providing a banding pattern resembling G-banding, facilitating identification of the chromosomes. (c) Hybridisation pattern of FITC labelled test DNA. (d)hybridisation pattern of the control DNA labelled with Texas-Red (e) CGH image comprising of red, green and blue colours (f) Colour ratios image, showing two chromosome 13 homologues as green confirming a gain of DNA materia corresponding to these chromosomes. Green Y chromosome and red X chromosome are the result of sex difference between the test (male) and control(female) DNA.

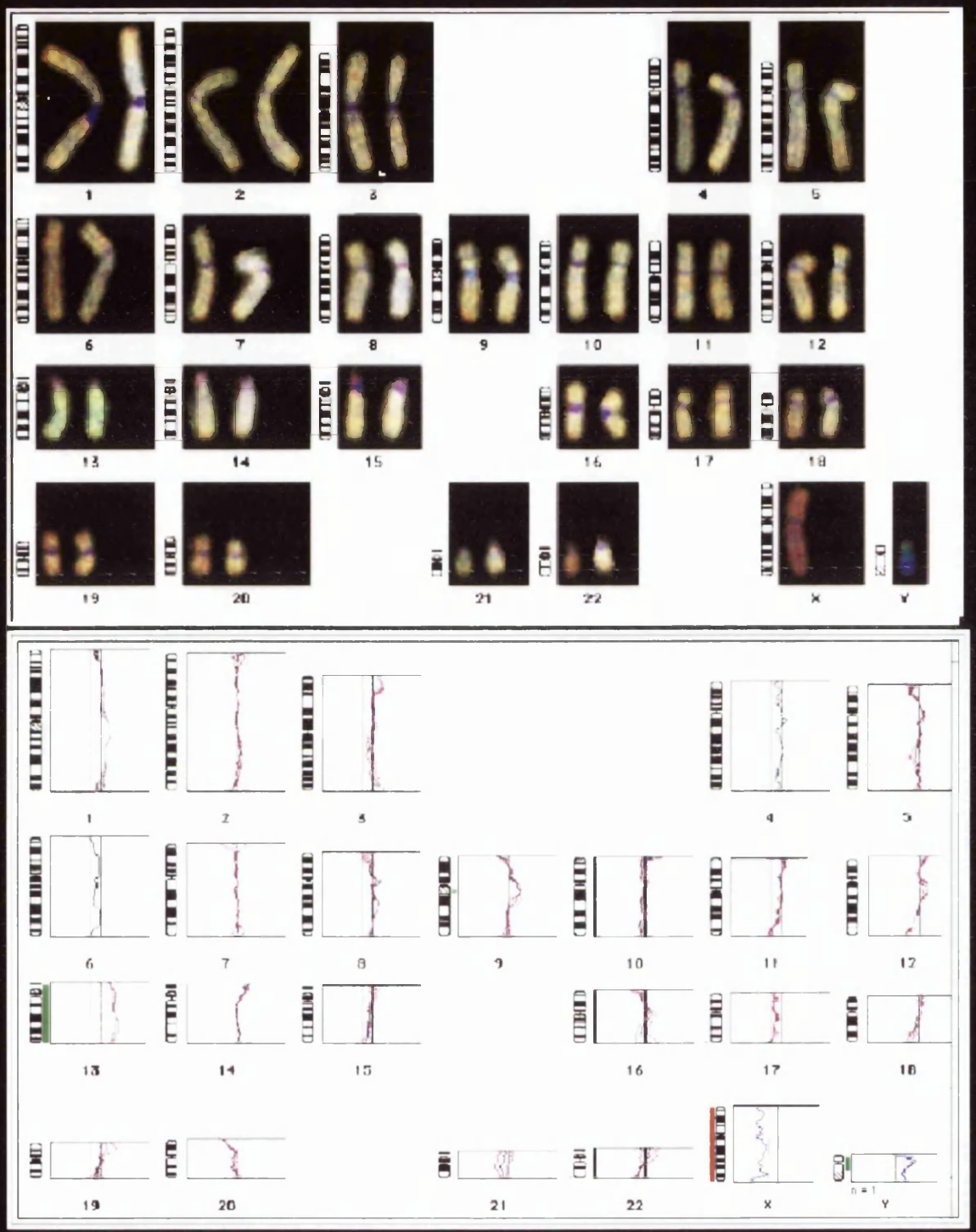


Figure 3. 7: Profile CGH analysis of the same case as Figure 3. 6. Mean green-to-red ratio profiles for chromosomes reflecting DNA sequence copy number changes. The three lines represent different levels of green-to-red ratios: middle-1.0 = no change in the test DNA.; left=0.50=loss; right 1.50, > 1.50= gain in test DNA. Gain or loss of DNA are also shown by green or red thick lines beside the idiograms. This ratio profile depicts the gains of DNA material corresponding to chromosome 13. Moreover, DNA corresponding to X and Y chromosomes are under and over-represented due to sex differences between the test (male) and control (female) DNAs.

3.3 Improving limits of CGH

3.3.1 CGH on a case with low percentage supernumerary marker chromosome

CGH was used to try to determine the origin of a mosaic ring chromosome present in a 71 year old woman with mental retardation. Repeated cytogenetic analysis had failed to identify the origin of the ring.

Earlier results

Cytogenetic

Cytogenetic studies by G-banding in 1976 had revealed the presence of two cell lines. Eighteen out of 25 cells showed a 46,XX karyotype while 7/25 (28%) showed an additional marker chromosome of unknown origin (46,XX/47,XX, + marker). Chromosome analysis by G-banding in 1986 had confirmed the finding of two cell lines. Twenty-one out of 25 cells examined showed apparently normal female karyotype while the remaining 4 cells (16%) showed a count of 47 including one additional small chromosome. The marker appeared to be a ring chromosome, approximately half the size of a chromosome 21, which contained at least one G-band positive area (Figure 3. 8 a). C banding had showed one centromere to be present, and Ag NOR staining was negative with respect to the ring chromosome. No other members of the family had undergone cytogenetic investigation.

FISH

FISH had been carried out using centromeric probes for chromosomes 4, 12, 20, 22, 13/21 and 1/5/19 empirically and failed to detect the origin of the extra chromosome. No further probes were investigated because difficulty had been experienced in identifying the marker with certainty within the metaphases.

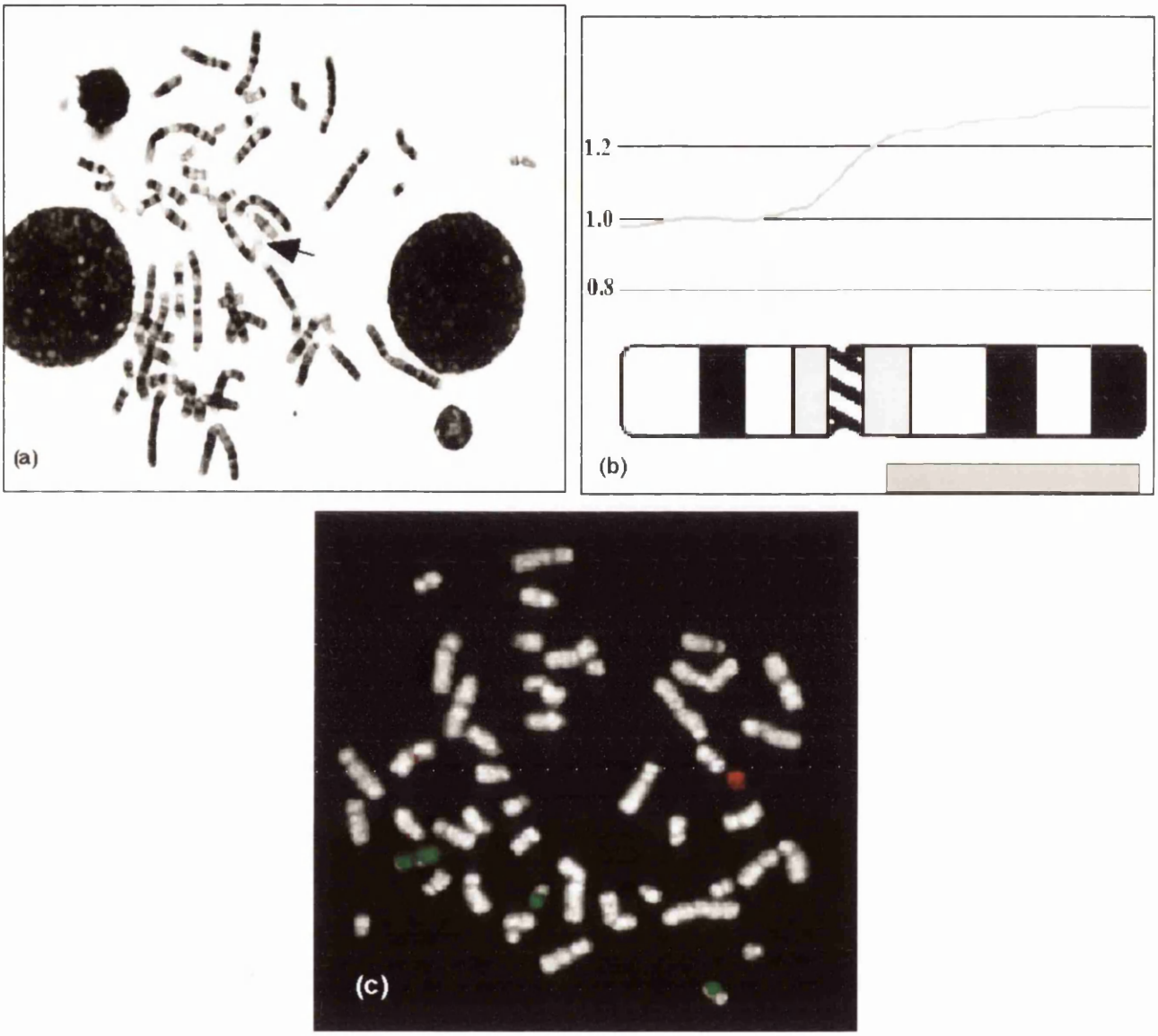


Figure 3. 8: (a) G-banded metaphase spread of the patient described in section 3.3 showing a supernumerary ring chromosome (arrowed). (b) Standard ratio profile of chromosome19 is demonstrated above the chromosome idiogram. The horizontal lines represent the balanced state of the chromosomal copy number (middle line, ratio 1.0) and the lower and upper thresholds applied to detect chromosomal losses and gains, respectively. These thresholds correspond to ratios of 0.8 and 1.20, which are the theoretical values expected for a monosomy or trisomy present in 40% of diploid cells. (c) Global CGH analysis showing gain of DNA material corresponding to 19q. Green X chromosome and Red Y chromosome are the results of the sex diffrence between test (female) and control (male) DNA.

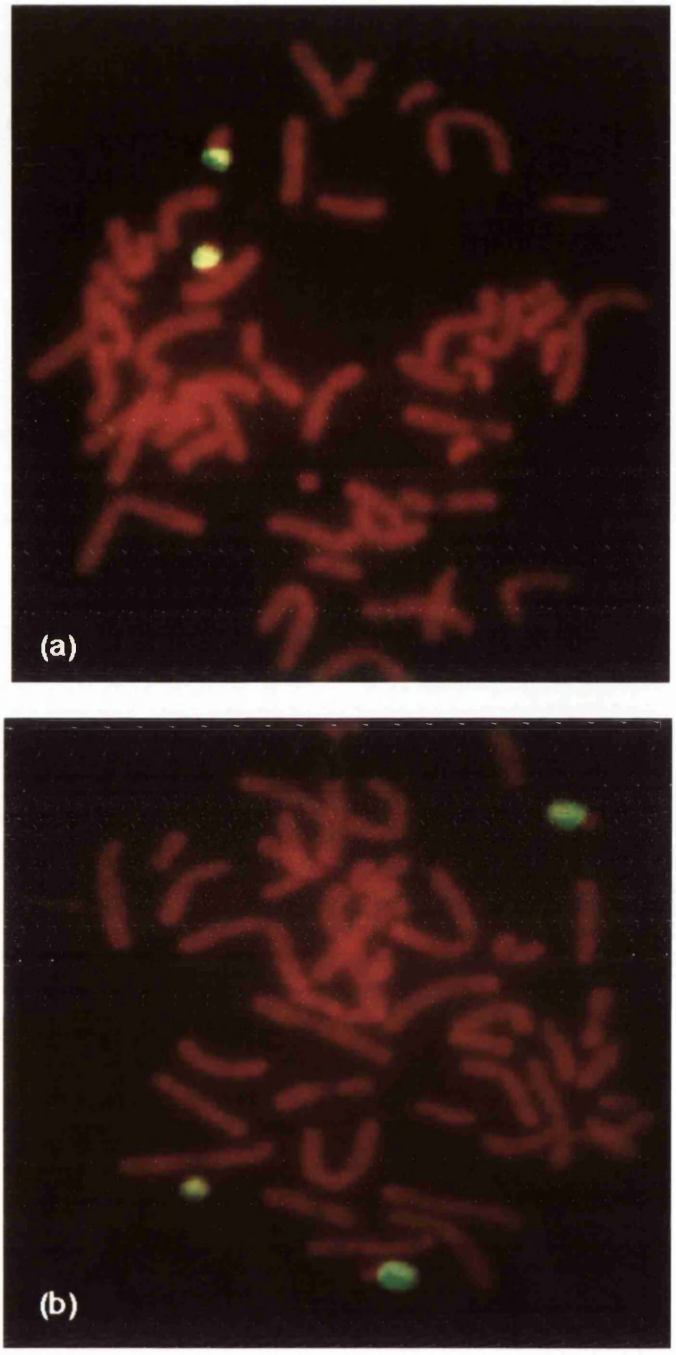


Figure 3. 9: FISH results of the case with marker chromosome using chromosome 19p (a) and 19q(b) arm specific painting probes. Chromosome 19p painting probe has hybridised to the 19p arm of both chromosomes 19 homologues but not to the marker chromosome. On the other hand 19q painting probe has hybridised to the long arms of both chromosomes 19 and the marker chromosome confirming the CGH results.

CGH Analysis

CGH was carried out using a DNA sample from the patient. Analysis using a very restrictive threshold with a fluorescence ratio of 0.5-1.5 (which requires 100% abnormal cells to detect any gain or loss of the DNA material) failed to detect any abnormality. Progressively less restrictive thresholds were then employed and, surprisingly, a threshold of 0.8-1.20 (which theoretically detects abnormal cells comprising 40% of the total) revealed a gain in DNA material corresponding to 19q (Figure 3. 8 b,c). In CGH, the centromeric areas are strongly suppressed by un-labelled human Cot-I DNA, so gain or loss of this area could not be interpreted reliably.

FISH confirmation of the CGH results

Further FISH studies using a whole chromosome 19 paint (WCP19, Oncor), 19p and 19q arm specific paints and telomeric probes for 19p and 19q (LI) and 19 centromeric probe confirmed the CGH finding. The ring chromosome hybridised to the whole chromosome 19 and 19q arm specific but not to 19p arm specific paint, 19p or 19q telomeric probes (Figure 3. 9-a, b). It was thus concluded that the proposita was trisomic for almost the entire long arm of chromosome 19 with only the telomeric region being absent from the ring.

3.3.2 CGH on samples with suspected cryptic abnormalities

3 families were carefully chosen for study. In each, two affected members with apparently normal karyotype and a similar phenotype were related through at least one healthy first degree relative. These features are highly suggestive of the segregation of a cryptic chromosomal translocation.

Detection of cryptic chromosomal translocations

Family 1

Patient JK was referred because of cranio-facial dysmorphism in association with tetralogy of Fallot, laryngomalacia and inguinal hernia. He died at the age of 6 months as a result of his cardiac abnormality. A paternal uncle who died in infancy from heart failure was reported to have had 'classical features of Noonan syndrome. The patient, although sharing some dysmorphisms associated with Noonan syndrome, did not have that condition but the close similarity to his uncle led to a suspicion that a cryptic translocation was segregating within the family. The results of chromosome analyses carried out on JK, his parents and other relatives in different service laboratories were all normal.

CGH analysis using standard approach

CGH analysis was performed on DNA from JK. Twenty metaphases with high-intensity uniform hybridisation were chosen for image analysis (see Methods). A standard type of CGH analysis including background correction and normalisation was performed using a fluorescence ratio (FR) threshold of 0.8-1.2. Average ratio profiles showed gains for the short arms of chromosomes 21 and 14 and the distal bands of chromosomes 17q and 1p. A loss of DNA material corresponding to the 2q telomeric area was also observed.

The application of a more restrictive threshold (FR: 0.5 - 1.5) narrowed the abnormal findings to chromosomes 17, 1 and 2. Using the more restrictive threshold in global CGH analysis of 20 metaphases, the same results were obtained. It was also observed that although the gain in chromosome 1p and deletion of 2q were consistent in most metaphases, they were found in only one homologue in each metaphase. On the other hand, the gain in 17q was consistently present in both homologues of most metaphases.

Table 3. 6 CGH results in JK using standard CGH approach with different fluorescence ratio (FR) thresholds

	FR threshold			
	0.8-1.2		0.5-1.5	
	Gain	Loss	Gain	Loss
Suggested abnormalities	1p, 14p, 17q 21p,	2q	1p, 17q	2q

CGH analysis based on the pattern of fluorescence intensity at chromosome ends

To investigate the significance of the results obtained by standard CGH analysis, the fluorescence intensity of red and green signals corresponding to test and control DNA in the 20 normalised CGH images was measured. Fluorescence intensity of the green signal (patient DNA) at both homologues of the 17q telomere, both before and after averaging, increased whilst that of the red signal corresponding to control DNA decreased (Figure 3. 10). At distal 2q, however, both before and after averaging, fluorescence intensity for green fell sharply but that of the red signal first slightly increased and then decreased, but at a slower rate than that of the green signal (Figure 3. 11). Quantification of the fluorescence intensities for both red and green at the 1p region showed decreasing patterns despite the ratio findings (Figure 3. 12).

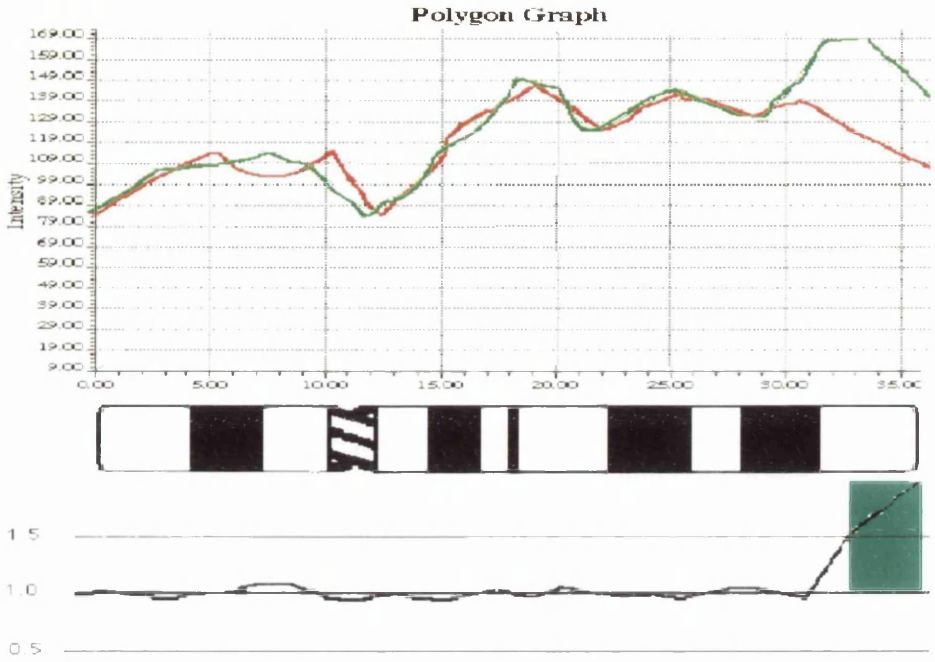


Figure 3. 10: Standard ratio profile of chromosome 17 is demonstrated below the chromosome idiogram. The three horizontal lines below the idiogram represent the balanced state of the chromosomal copy number (middle line, ratio 1.0) and the lower (lower line) and upper (upper line) thresholds applied to detect chromosomal losses and gains, respectively. These thresholds correspond to ratios of 0.5 and 1.5, which are the theoretical values expected for a monosomy or trisomy present in 100% of diploid cells. The polygon graph above the idiogram shows the fluorescence intensity along chromosome 17. As shown, while at the distal p-arm both colours are decreasing as usual, at the distal q-arm the intensity of fluorescence is increasing for the green signal and decreasing for the red. This shows that the decreasing effect of fluorescence at the telomere is compensated by the extra copy of the DNA at this region.

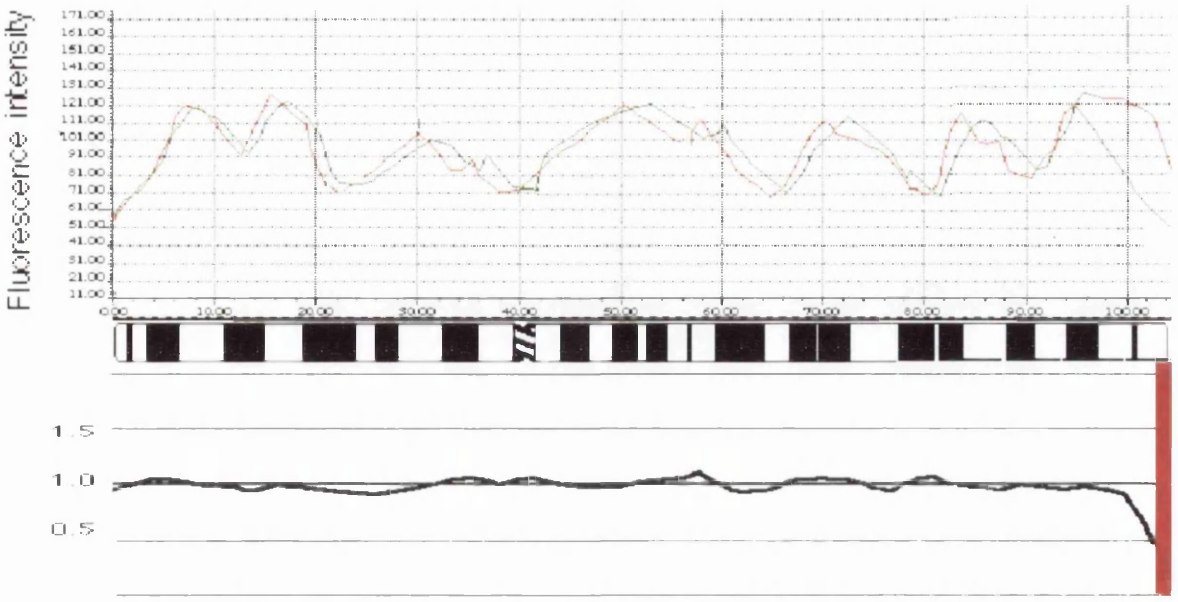


Figure 3. 11: Standard ratio profile of chromosome 2 (below the idiogram) shows a loss of DNA material related to the last band on 2q. The polygon graph (above the idiogram) demonstrates the different gradients for fluorescence intensity secondary to the DNA copy loss at this particular area. At the p-arm, the fluorescence intensity for both green and red are following the usual decreasing pattern of the chromosomal ends.

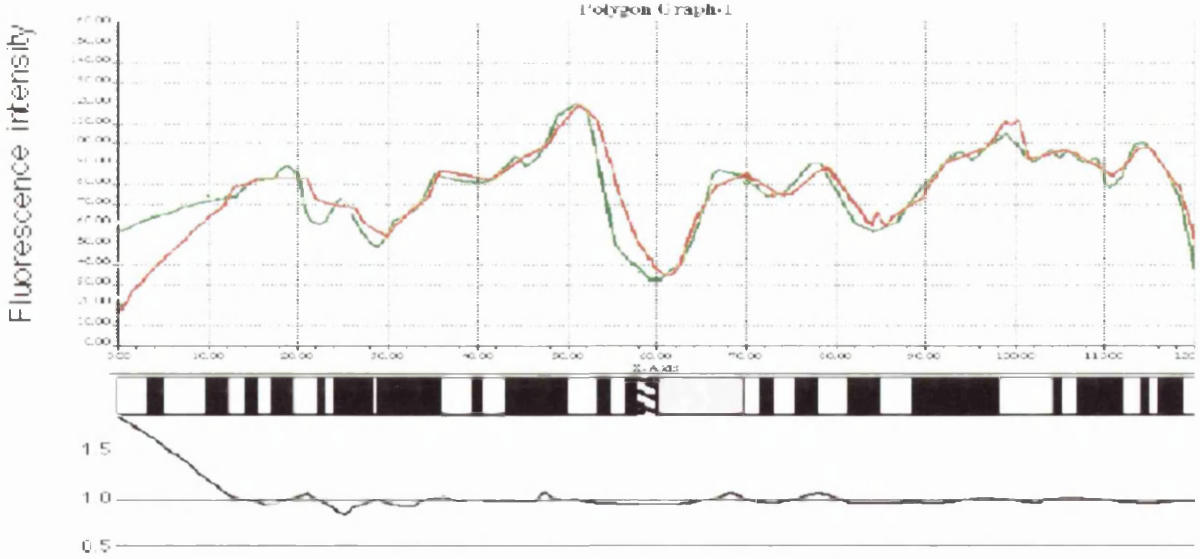


Figure 3. 12 : Standard ratio profile of chromosome 1 (below the idiogram) shows a gain of DNA material related to the last band on 1p. However, fluorescence intensity quantification does not support this finding (by decreasing for both colours).

To rule out sources of error, the CGH experiment was repeated with the fluorochromes reversed (red for test and green for control DNA). Various different control samples were used including DNA from both parents and several unrelated individuals. Under all these conditions the findings for chromosomes 17 and 2 remained consistent. These results suggested strongly that there was at least one extra copy of chromosome 17q telomeric region, and if the patient had an unbalanced translocation, the extra material was most likely translocated to the distal end of 2q, leading to partial monosomy/trisomy for chromosomes 2q and 17q.

As expected for chromosomally balanced karyotypes, CGH analysis showed no gain or loss of DNA material in any relative connecting the patient and his deceased uncle, nor were there any CGH abnormalities in other healthy family members.

Confirmation of CGH results by FISH

To confirm the CGH findings, FISH was carried out on metaphase chromosomes from JK using commercial probes specific for the telomeric regions of chromosomes 17q (Oncor) and 2q (LI). Three signals for 17q telomere were detected, one on each 17q, and the third signal was translocated to a 2q telomere (Figure 3. 13-a). Conversely, the chromosome 2q telomeric probe showed only one signal which was located normally on one homologue of chromosome 2 (Figure 3. 13-a).

Using the same FISH probes on the other members of the family, JK's father and grandfather were found to be carriers of the balanced translocation (Figure 3. 13-b), while the mother and grandmother had normal results.

The size of the abnormality deduced from averaged relative length of each chromosome to total haploid autosome was estimated to be ~10 Mbs for the duplication of 17q and ~4 Mbs for the 2q deletion (see Figure 3. 10).

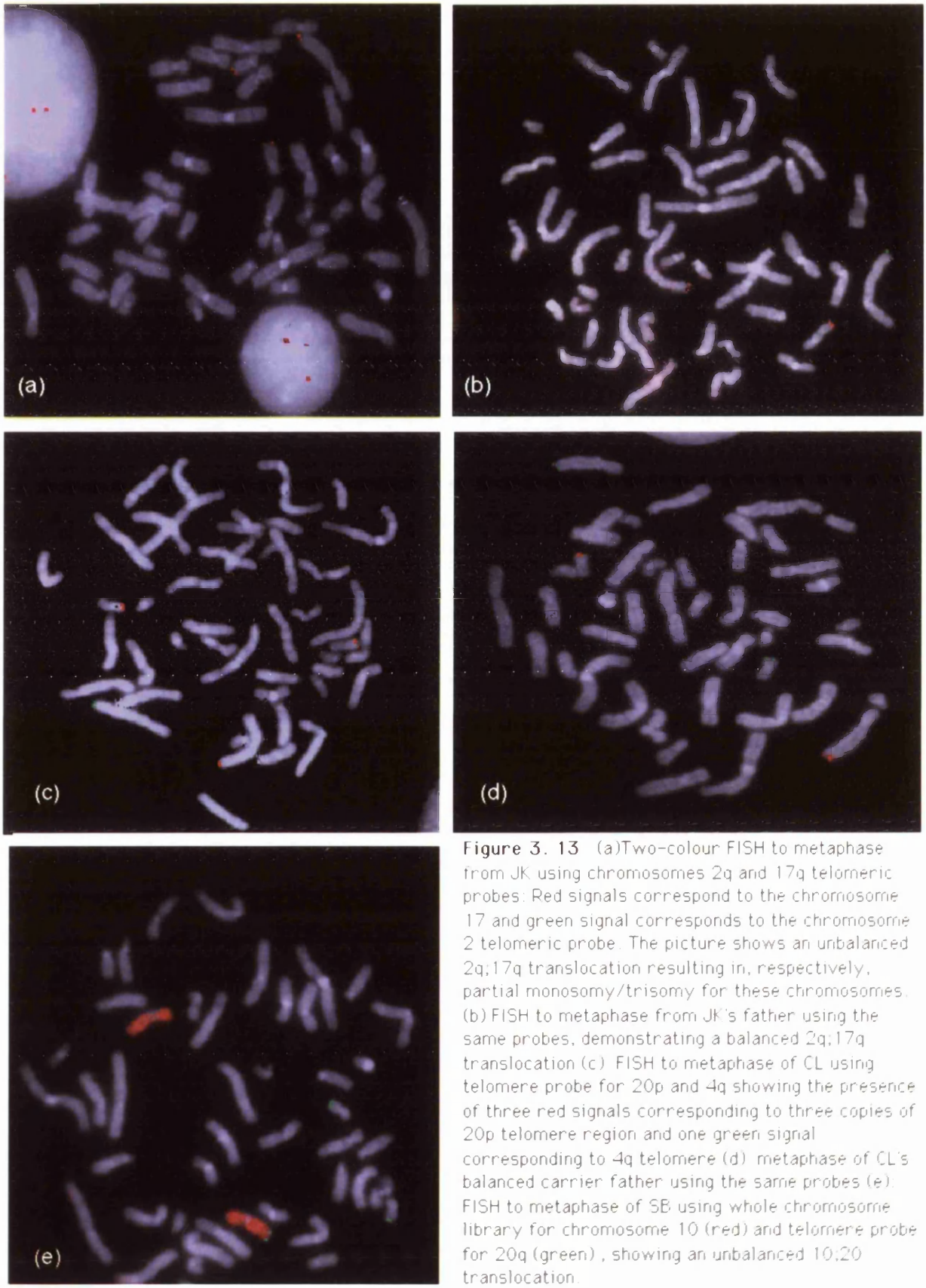


Figure 3. 13 (a)Two-colour FISH to metaphase from JK using chromosomes 2q and 17q telomeric probes. Red signals correspond to the chromosome 17 and green signal corresponds to the chromosome 2 telomeric probe. The picture shows an unbalanced 2q;17q translocation resulting in, respectively, partial monosomy/trisomy for these chromosomes. (b) FISH to metaphase from JK's father using the same probes, demonstrating a balanced 2q;17q translocation (c) FISH to metaphase of CL using telomere probe for 20p and 4q showing the presence of three red signals corresponding to three copies of 20p telomere region and one green signal corresponding to 4q telomere (d) metaphase of CL's balanced carrier father using the same probes (e) FISH to metaphase of SB using whole chromosome library for chromosome 10 (red) and telomere probe for 20q (green) , showing an unbalanced 10;20 translocation.

Family 2

Patient CL, a 32 month old girl was referred with mild motor and speech delays, a small ventricular septal defect and minor facial dysmorphisms including low set ears, a flat philtrum and a wide mouth. In addition, she had bilateral vesico-ureteric reflux (VUR). In the family history, a male first cousin had identical, mild developmental delays, minor facial dysmorphism and had undergone surgery for VUR. Both cousins and their parents had normal G-band karyotypes (400-500 band level).

DNA from CL was the subject of CGH analysis using the modified technique and this suggested gain of 20p telomere and loss of 4q telomere. The size of the abnormality was estimated to be 7Mbs for the 20p duplication and 10Mbs for the 4q deletion. The results of FISH analysis using the specific telomeric probes for these chromosome regions [20p (Oncor) 4q (LI)] confirmed the CGH results, identifying an unbalanced translocation between the telomeric bands of chromosomes 4q25 and 20p13. Both affected cousins had partial trisomy for chromosome 20p and partial monosomy for chromosome 4q. In turn, the mother of one affected cousin and the father of the other were found to carry a balanced reciprocal 4q; 20p translocation which was inherited from their own mother (Figure 3. 13: c,d).

Family 3

Patient SB, a first-born male infant, was referred to the clinical genetic department at age 2 years when moderate mental handicap was diagnosed. He had no major congenital abnormalities and minimal dysmorphism but in the family history his maternal aunt, FM, had severe, unexplained mental handicap. This 32 year-old lady was examined and found to have short stature (height 140 cm), microcephaly (OFC 51cm) and non-specific facial dysmorphisms. In view of this family history, high-resolution chromosome analyses were repeated on both patients and their relatives on several occasions and although no abnormality was detected, an underlying cryptic chromosome translocation was always suspected on clinical grounds.

Modified CGH was performed on a DNA sample from FM and showed partial gain and loss of DNA material corresponding to the distal bands of chromosome 20q and 10q respectively. The size of the duplicated 20q was estimated to be 5 Mbs and loss of 10q region was about 10 Mbs. FISH studies using 10q and 20q telomeric probes [LI] confirmed partial trisomy for 20q and partial monosomy for 10q. The same unbalanced abnormality was also detected in SB (Figure 3. 13). The balanced reciprocal translocation was detected by FISH in SB's mother and maternal grandfather, but it was not present in a maternal aunt of SB.

Further CGH on suspected cases of cryptic abnormalities

Samples from affected members of 8 more families were the subject of CGH studies using the modified approach. However, CGH did not detect any abnormalities in the investigated families.

3.3.3 CGH on a sample with hypotriploidy

A case of rhabdomyosarcoma was the subject of conventional chromosome analysis and comparative genomic hybridisation. Cytogenetic analysis revealed the presence of four cell lines. There were three abnormal hypotriploid clones with the following karyotypes:

60,XY,+2,+3,+5,+8,+11 ,+13,+13,+13,add(17) (q25) ,+19,

+20,+21,+21,+22,+22/

63,XY,+1 ,+2,+3,+5,+8,+8,+11,+11 ,+marker [?del (12)(q11 1q15)],

+13,+13,+13,add(17)(q25),+20,+21,+21,+22,+22/

64,XY,+1 ,+2,+3,+5,+8,+8,+11,+11 ,+marker [? del (12)(q11 1q15)],

+13,+13,+13,add(17)(q25),+19,+20,+21,+21,+22,+22/

46,XY

A DNA sample extracted from the tumour was the subject of CGH analysis.

Standard CGH analysis based on the fluorescence ratio value from the whole metaphase showed loss of DNA in chromosomes, 4, 6, 7, 9, 10, 14, 15, 16,

17, 18,X and Y. Gain of DNA material corresponding to chromosome 13 was also observed (Figure 3. 14). No gain or loss of DNA corresponding to chromosomes 1, 2, 3, 5, 8, 11, 12, 13, 19, 20, 21 and 22 was detected.

When this result was compared with that of cytogenetic analysis it was clear that the trisomic chromosomes were being interpreted as normal and the disomic chromosomes as loss. Based on the G-banding results, chromosomes with known ploidies were then identified and used as index chromosomes in the next CGH analysis. In a global CGH analysis approach using SmartCapture package, the index chromosomes were identified and the red and green values along the chromosome axis were measured and normalised by equalising the fluorescence intensities. The green to red ratios along the remaining chromosomes were then calculated.

In first series of image analysis chromosomes 4, 6, 7, 9, and X (which were disomic by G-banding) were selected as index chromosomes. No loss of DNA was detected and DNA sequences corresponding to chromosomes 1, 2, 3, 5, 8, 11, 12, 13, 20, 21 and 22 were over represented (Figure 3. 15). When chromosomes 1, 2, 3, 5 and 20 (where G-banding had shown 3 copies of each) were used as index chromosomes all disomic chromosomes were under represented while chromosome 12 showed no gain or loss compared to the index chromosomes. On the other hand chromosomes 8, 11, 13, 21 and 22 showed higher copy numbers than the index chromosomes (Figure 3. 16). Finally, chromosomes 8, 11, 21 and 22 were selected as index chromosomes, and all non-index chromosomes except 13 were under represented, being the only chromosome that showed higher copy number than the index chromosomes (Figure 3. 17). These results confirmed the G-banding analysis and the suggestion that the marker chromosome originated from chromosome 12. However they did not detect any deletion of DNA material in the third copy of chromosome 12 nor show any gain of DNA in chromosome 17. Based on the findings of this modified approach and the chromosome analysis results it can be suggested that the additional material on chromosome 17 originated from chromosome 12. As more material was not available, further confirmatory FISH was not possible.

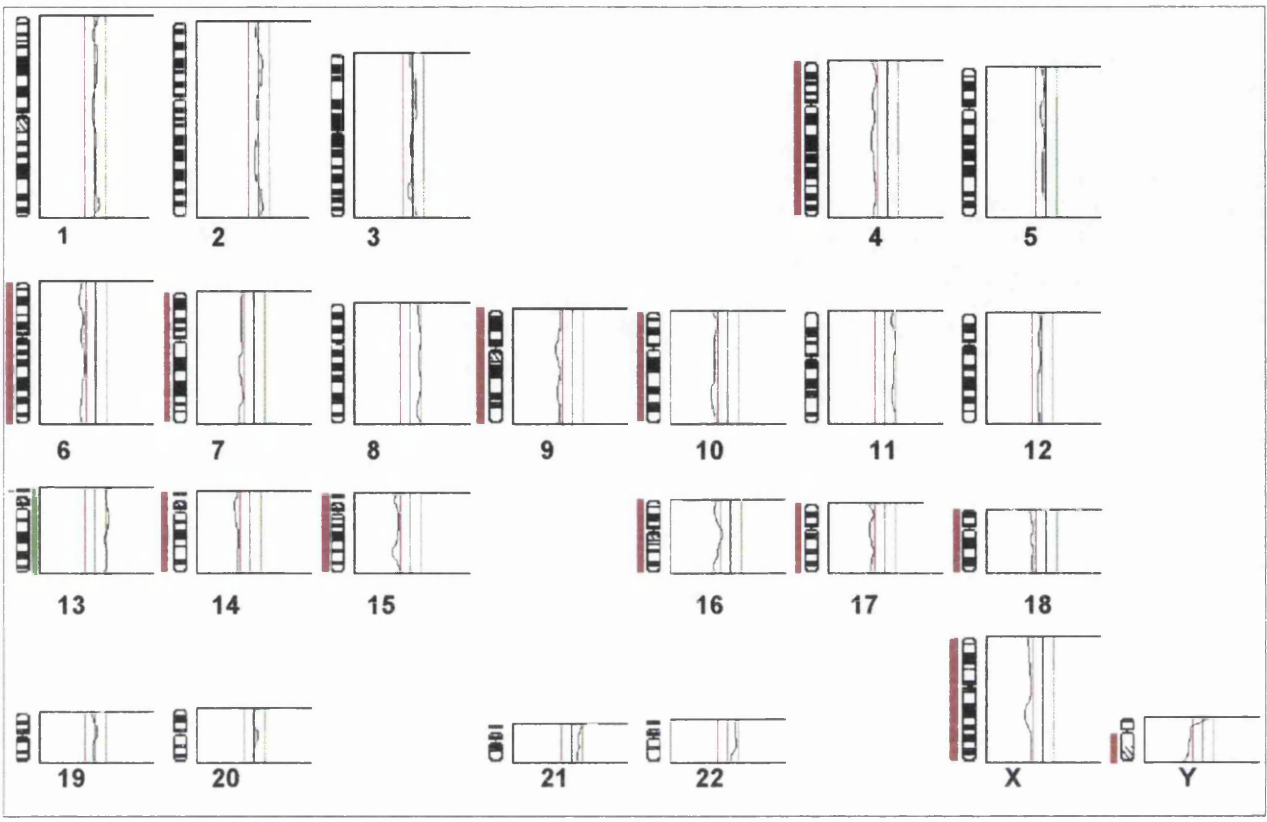


Figure 3. 14: Standard CGH ratio profile from the case with hypotriploidy. Disomic chromosomes (see text) are under-represented (red thick lines beside the idiograms) while chromosomes with 3 or 4 copies are represented as normal. Chromosome 13 which had 5 copies in G-banding is over-represented here (thick green line beside the idiogram).

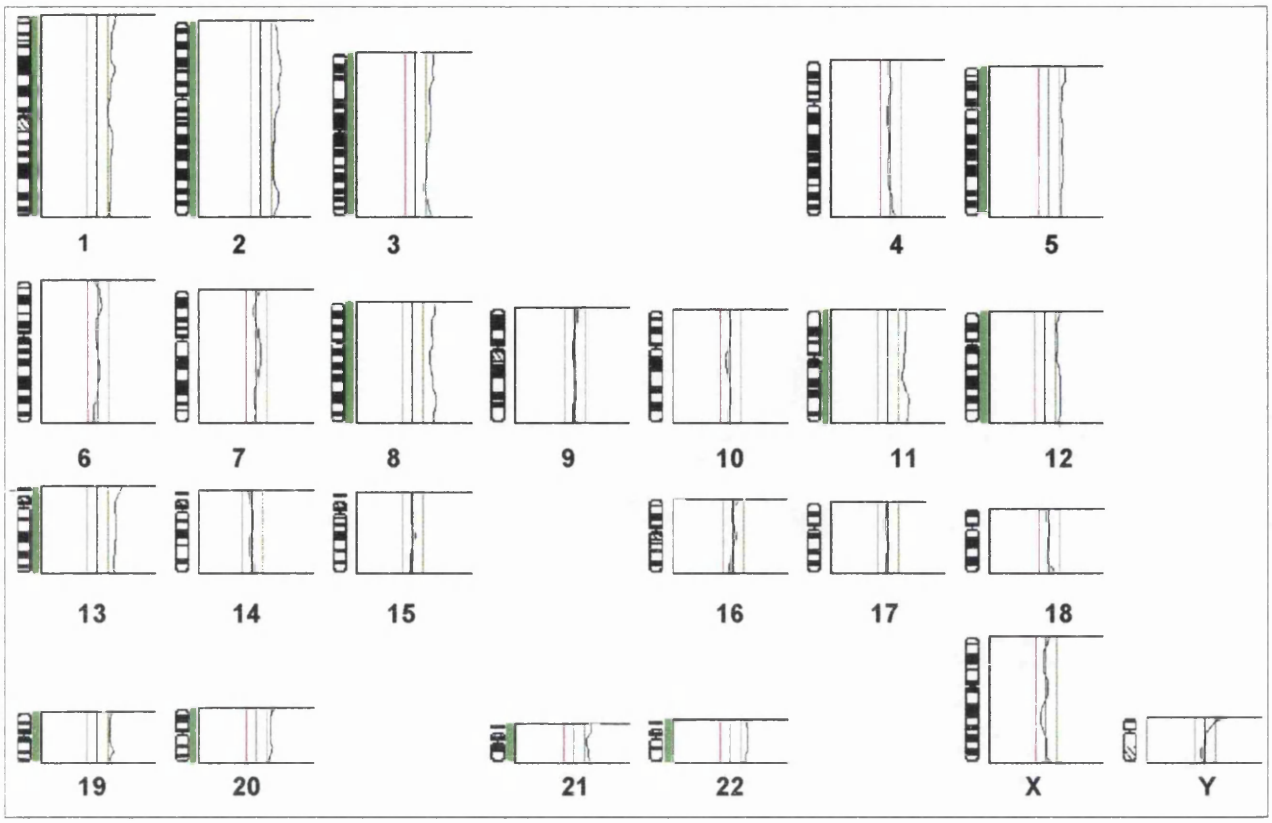


Figure 3. 15: CGH analysis by modified approach. Disomic chromosomes are selected as index and all other chromosomes are analysed based on them. As shown, all chromosomes with 3, 4, or 5 copies are over-represented here (green lines beside idiograms).

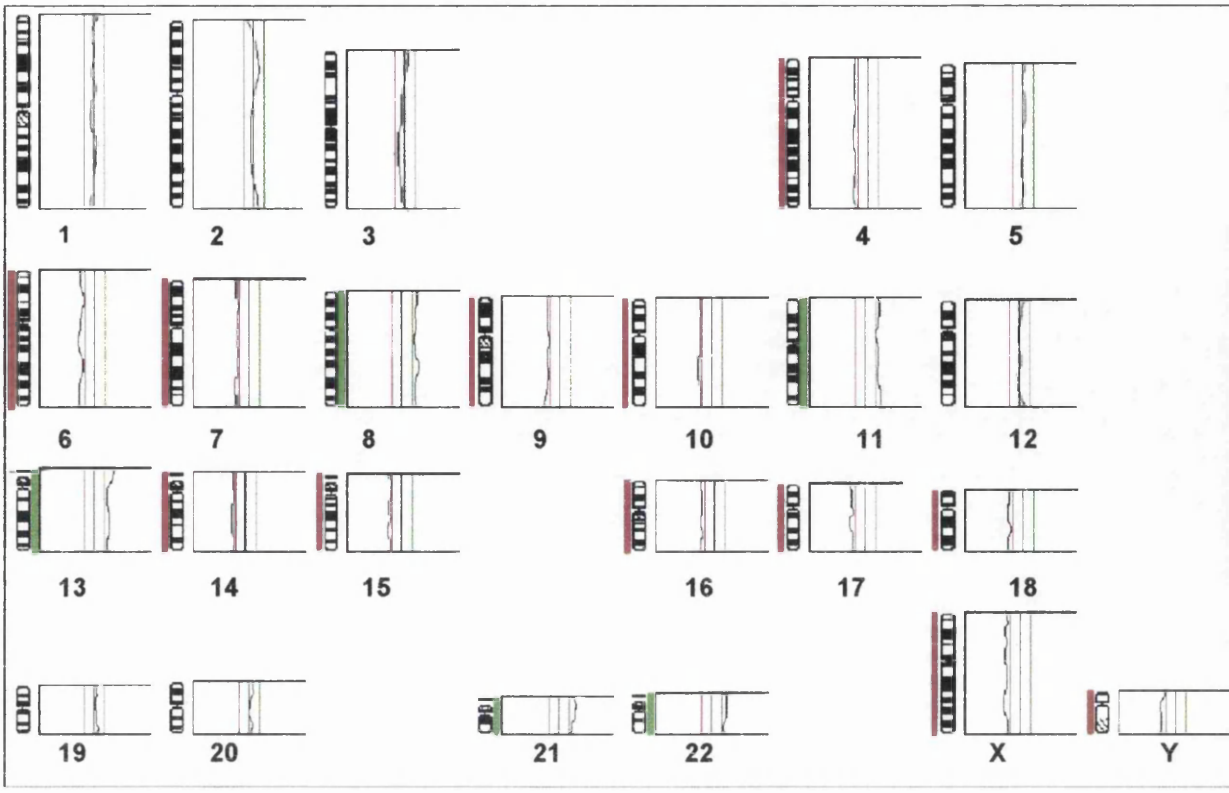


Figure 3. 16: CGH ratio profile from the case with hypotriploidy using the modified analysis. CGH image is normalised based on chromosomes with 3 copies. Disomic chromosomes (see text) are under-represented (red thick lines beside the idiograms) while chromosomes with 3 or 4 copies are represented as normal. Chromosome 13 which had 5 copies in G-banding is over-represented here (thick green line beside the idiogram).

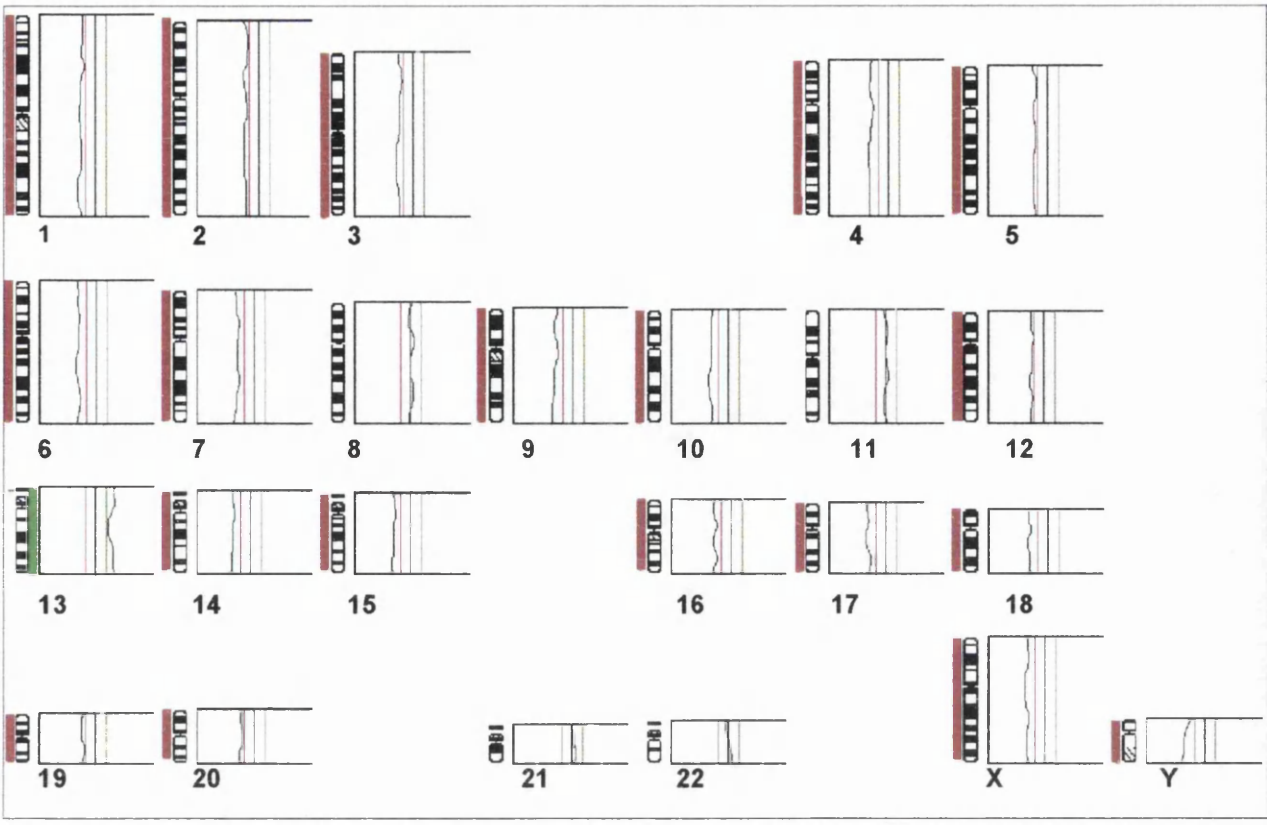


Figure 3. 17: CGH ratio profile from the case with hypotriploidy using the modified analysis. CGH image is normalised based on chromosomes with 4 copies. Disomic and trisomic chromosomes (see text) are under-represented (red thick lines beside the idiograms) while chromosomes with 4 copies are represented as normal. Chromosome 13 which had 5 copies in G-banding is over-represented here (thick green line beside the idiogram

3.3.4 ***Overnight CGH***

Samples from 19 cases with normal karyotypes or various numerical chromosome abnormalities were used. A modification of the routine protocol which included five micrograms of test and control DNAs, 5 nmoles of each fluorochrome and 180 minutes nick translation time, successfully detected unbalanced abnormalities in all patients. Parameters modified for overnight CGH are compared with those used in 48-72 hour protocol (Table 3. 7).

Table 3. 7: Overnight CGH parameters compared with those of standard CGH protocol

	DNA µg /experiment	Fluorochrome mmole/experiment	Nick translation time (minutes)	Cot-I DNA µg /experiment
48-72 Hour Method	1	1	55	50
Overnight Method	5	5	180	30

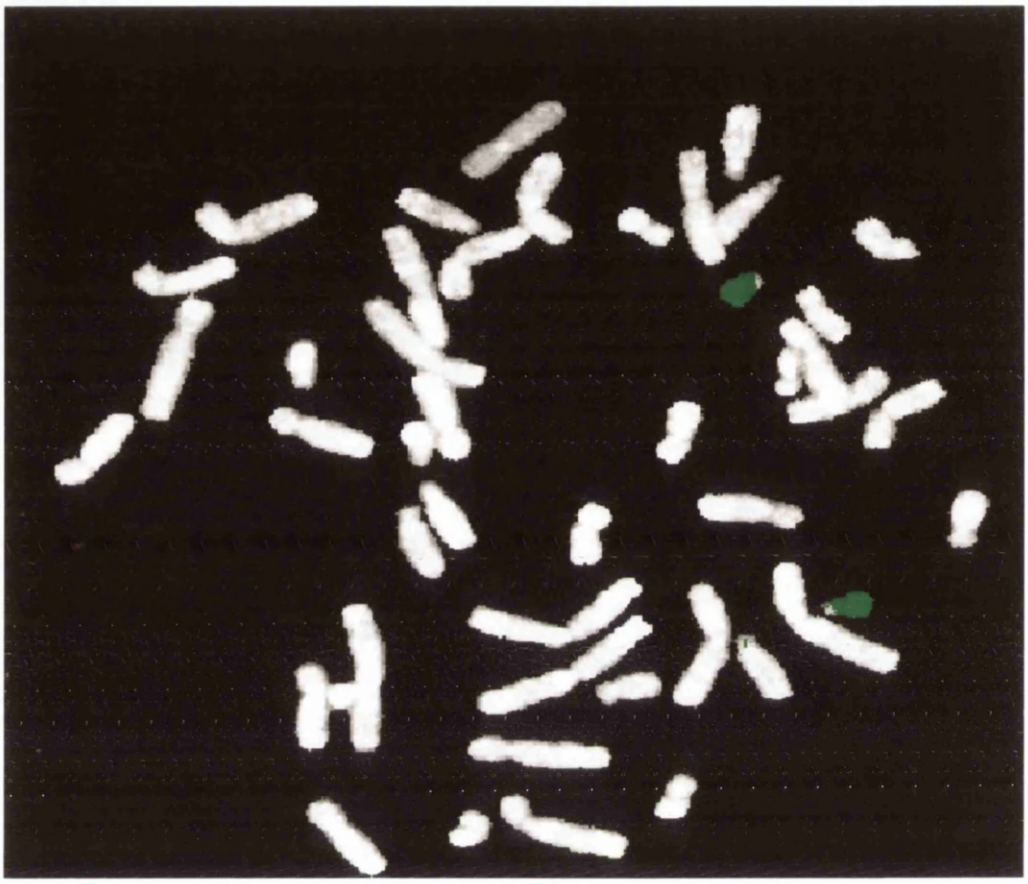


Figure 3. 18: Global CGH result of a case with trisomy 21 studied by overnight CGH approach. Test (green) and control (red) DNA were from the same sex. Both chromosome 21 homologues are coloured in green confirming the gain of DNA material corresponding to these chromosomes in the test DNA compared to that of the control DNA.

3.4 Reinterpretation of a putative isochromosome Xp

Extensive cytogenetic, molecular cytogenetic and molecular studies were performed on samples from a female patient with mental retardation and karyotype 46,X?iXp. Comparative genomic hybridisation, however, revealed the presence of chromosomal segments corresponding to proximal Xq. Quantitative PCR (see Methods), by targeting *XIST* gene, confirmed the CGH results and showed that the X inactivation centre locus had remained intact in the abnormal chromosome X. The karyotype was finally shown to be: 46, X, t(X;X)(p11;q21).

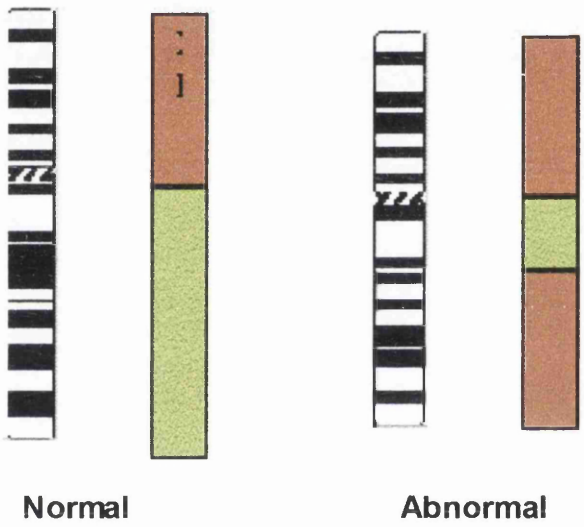


Figure 3. 19: Schematic representation of the chromosome abnormality in the case presented in 3.4. As shown four fifth of the q arm of the abnormal X is deleted and replaced with the p arm resulting in a partial trisomy/monosomy for the Xp and Xq respectively.

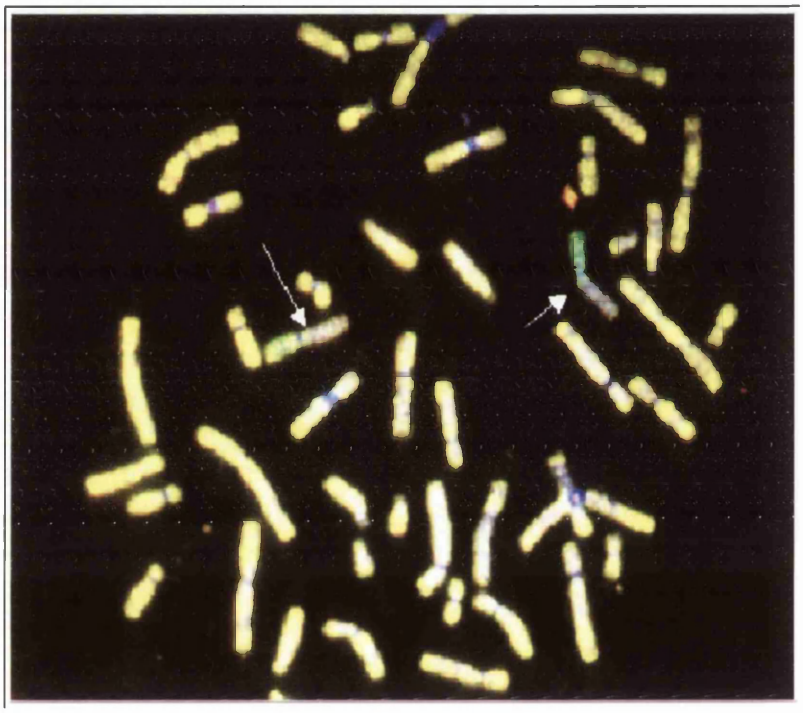


Figure 3. 20: CGH image using a DNA sample from the case with suspected iXp. This image shows signals on the proximal region of Xq with the higher intensity than the remaining xq, ruling out the complete deletion of Xq.

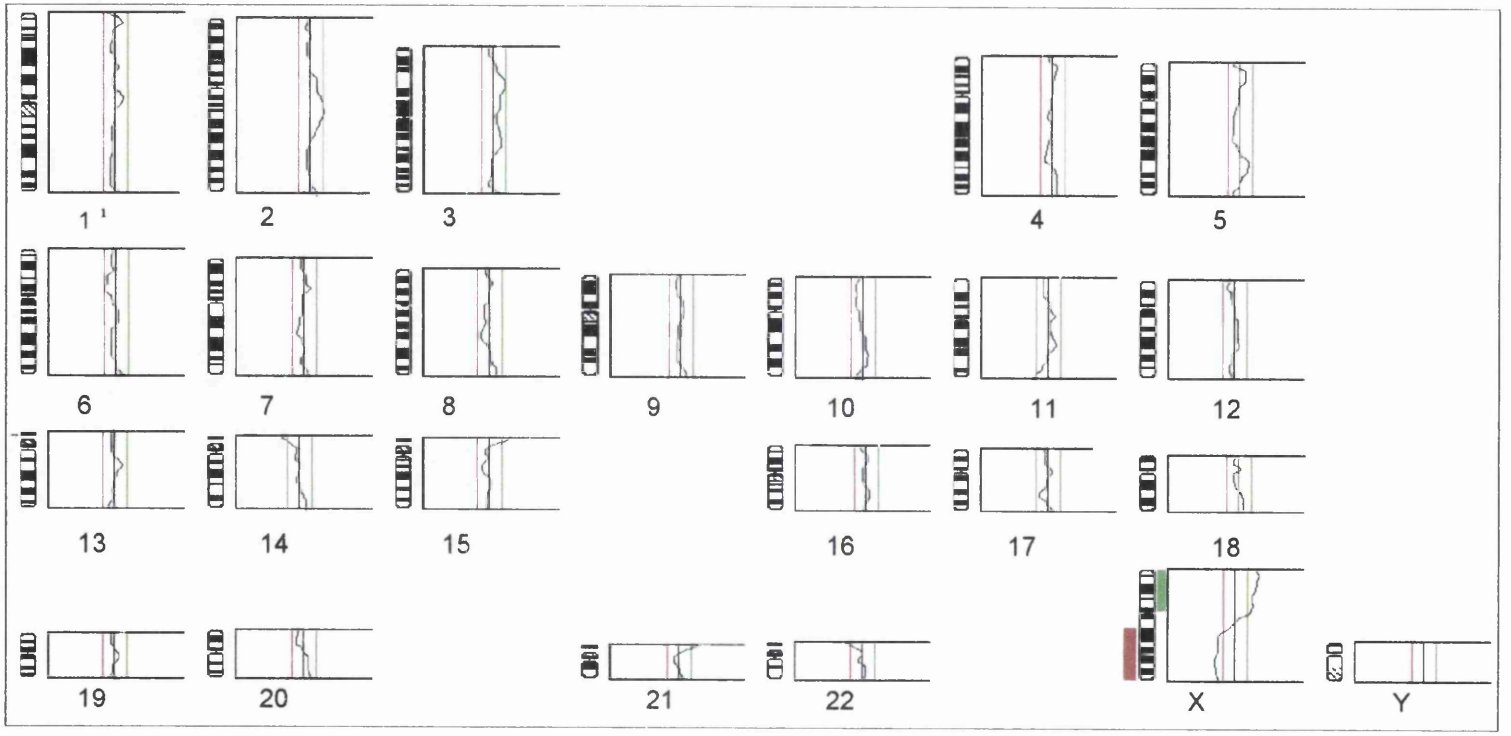


Figure 3. 21: Profile CGH analysis showing a deletion of 4/5 of Xq and gain of whole Xp.

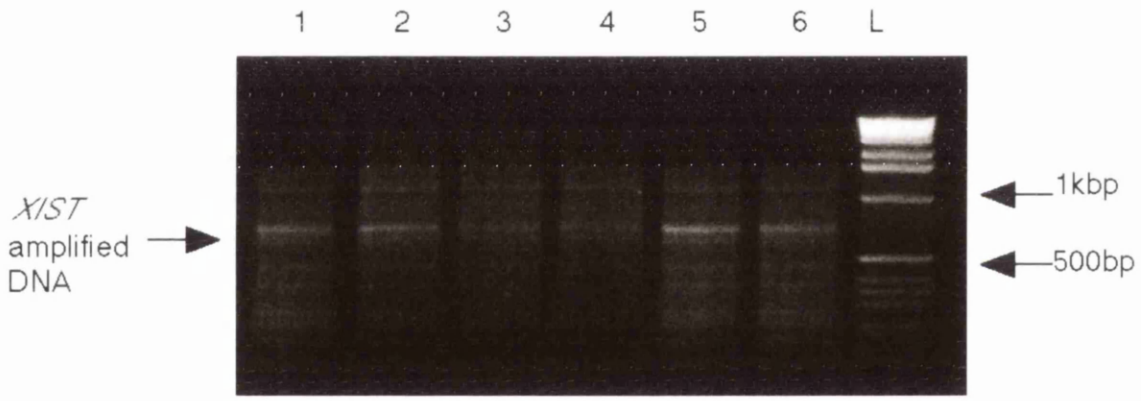


Figure 3. 22: Quantitative PCR results from the case described above targeting the *XIST* gene. 1-2= patient, 3-4=normal male, 5-6= normal female, L= ladder. PCR product from the patient is in the same size and intensity as that of the normal female and more than that of the normal male. This visual finding is also confirmed with the digital measurement using IP-lab image analysis software.

3.5 A case of follicular lymphoma with complex chromosome abnormalities

Conventional chromosome analysis

G-banding chromosome analysis of direct preparations of lymph node from the patient revealed the presence of two clones.

The majority of cells had a male karyotype with duplications of part of the long arms of chromosomes 1 and 12 and a deletion in the long arm of chromosome 14. There was an apparently balanced translocation between the long arms of chromosomes 19 and 22 and a derivative chromosome 3 with additional material of unknown origin. There were also around ten double minutes in every cell. The second clone had all the abnormalities of the first but had another copy of the duplicated chromosome 1 replacing the normal homologue found in the first clone.

CGH analysis

CGH analysis was performed using a DNA sample extracted from the lymph node from the patient. Gains of DNA material corresponding to 1q12-25, 12q13-21, and 8q22-ter together with a loss of DNA corresponding to 14q24-ter were detected. Moreover, an amplification of DNA corresponding to distal 3p was also observed.

Based on these results, it was suggested that the additional material on chromosomes 1 and 12 were the result of duplication. On the other hand, as both chromosomes 8 in G-banding were normal, the CGH finding of gain of 8q was suggested to be from the extra material on the 3p. Finally, the double minutes were suggested to be the product of amplification of terminal 3p.

Figure 3.23: G-banded metaphase chromosomes with multiple abnormalities from the case with follicular lymphoma. Some double minutes are indicated by arrows.

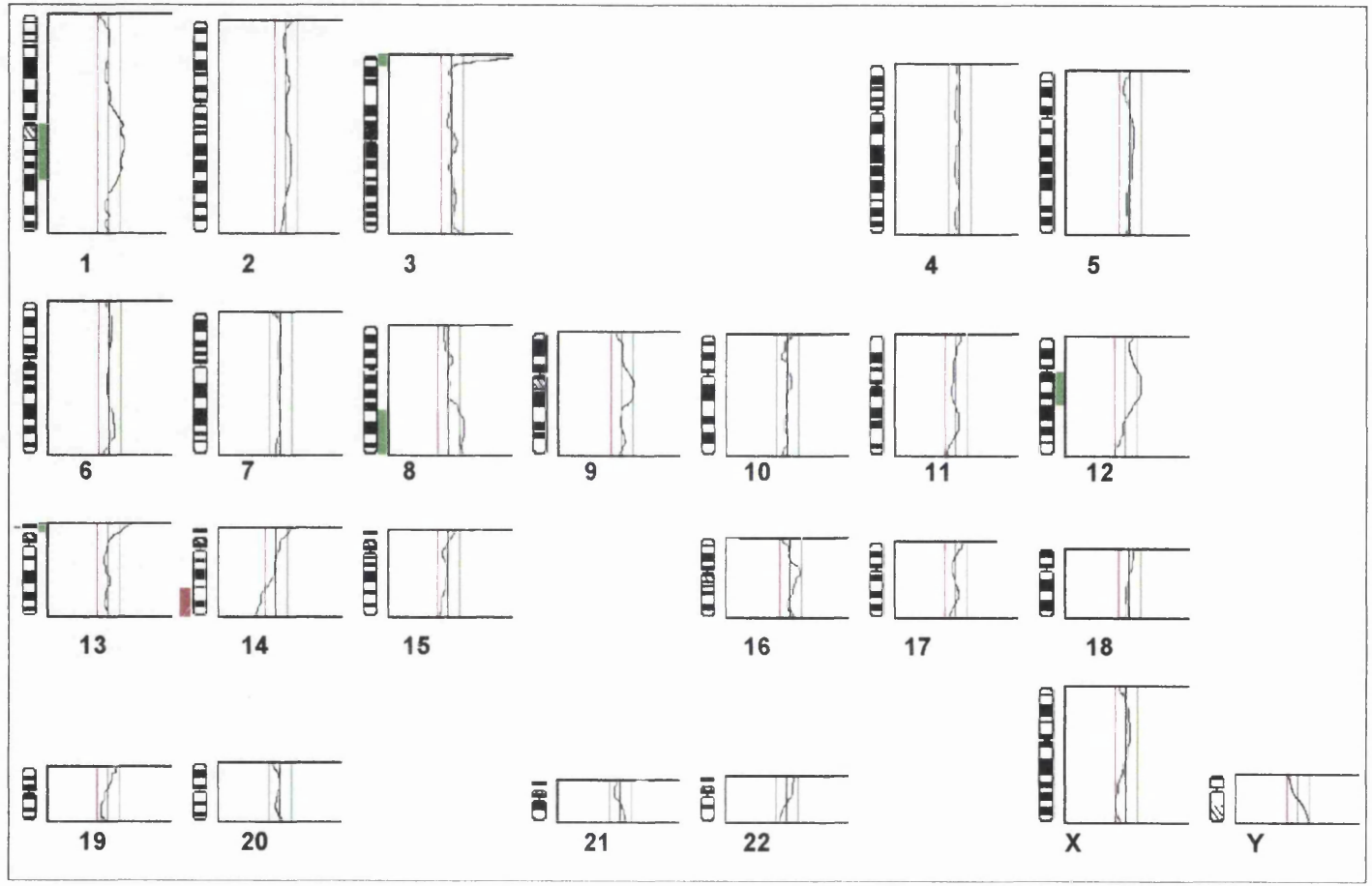


Figure 3. 24: Mean green-to-red ratio for profiles for chromosomes reflecting DNA sequence copy number changes in the case presented in 3.5. The three lines represent different levels of green-to-red ratios: middle-1.0 = no change in the tumour DNA.; left=0.50=loss; right-1.50, > 1.50= gain in tumour DNA. Gain or loss of DNA are also shown by green or red thick lines beside the idiograms. This ratio profile depicts the gains of DNA material at 1q12-25, 12q13-21, and 8q22-ter, together with a loss of DNA corresponding to the region14q24-ter. Moreover, an amplification of DNA corresponding to distal 3p is also clear.

FISH confirmation of CGH results

FISH, using painting probes from chromosomes 1, 3, 8, and 12 was carried out.

Chromosomes 1 and 12 painting probes hybridised to the whole of both chromosomes 1 and both 12 confirming the CGH suggestion of duplication at 1q and 12q.

Chromosome 3 painting probe hybridised to all double minutes, the whole of the normal 3 and a part of the short arm and the whole of the long arm of the abnormal chromosome 3. However, it did not hybridise to the additional material on the abnormal chromosome 3. Chromosome 8 painting probe hybridised to both normal chromosome 8s and to the additional material on chromosome 3 (Figure 3.25 and Figure 3. 26).

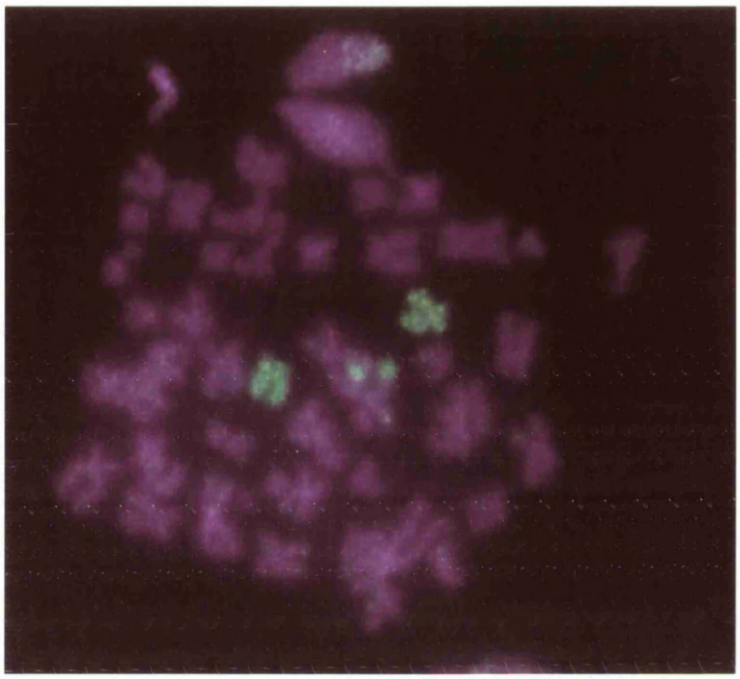


Figure 3.25: FISH using chromosome 8 painting probes: probes are hybridised to both chromosome 8s together with the additional material at the distal 3p, confirming an unbalanced 3;8 translocation.

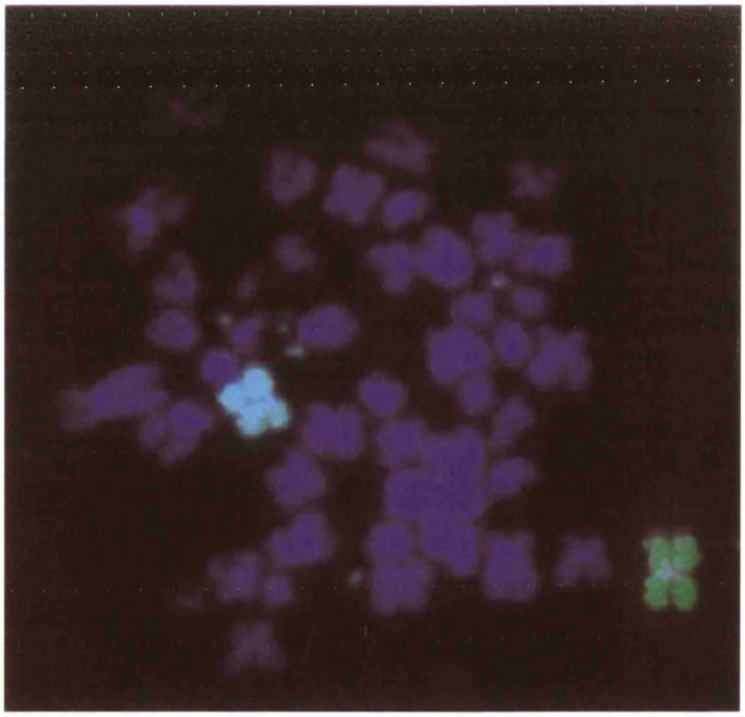


Figure 3.26: FISH using chromosome 3 painting probes: probes are hybridised with the whole of the normal homologue of chrmosome 3 and most part of the abnormal 3. Additional material at the distal 3p is not hybridised with chromosome 3 painting probe. Chromosome 3 painting probes are also hybridised to all double minutes confirming that the double minutes are originated from chromosome 3.

3.6 CGH in haematological malignancies

Comparative genomic hybridisation was used to investigate the assignment of chromosomal re-arrangements in blood or bone marrow samples of patients with haematological malignancies.
Fourteen cases were examined (7 cases with AML, 3 cases with CML, 2 cases with ALL, 1 case with CLL and 1 case with MDS). All CGH experiments were performed in a blind manner in parallel to conventional cytogenetics.

Volume of samples

DNA was extracted from material remaining after routine cytogenetic cultures had been set up. This was almost invariably less than 1 ml of sample, and in 9 cases a wash out of the sample tube was all that was available. However, results were obtained from all samples.

Comparison of CGH results with G-banding results

In 2 cases CGH did not detect the abnormality found by conventional cytogenetics because of the balanced nature of the rearrangements. Both CGH and conventional cytogenetics found no abnormality in 5 samples. In 3 abnormal cases CGH and G-banding results were compatible. Complete CGH and banding data are listed in Table 3. 8.

A case of ALL with 9p;9q translocation

In a case of ALL which had been reported as having a normal karyotype, CGH detected a concurrent loss of 9p and gain of 9q material. This finding could not be confirmed on the original sample by FISH, due to insufficient material. However, one year later, when the patient had a relapse, FISH on metaphase chromosomes prepared from a lymph node sample confirmed the CGH finding. Chromosome 9q arm painting probe hybridised to the whole q arms of the both normal and abnormal chromosome 9 and almost the whole of the p arm of the abnormal chromosome 9. Chromosome 9p arm specific

painting probe hybridised to the whole of the p arm of the normal chromosome 9 and a small segment immediately adjacent to the centromere of the abnormal chromosome 9. FISH results thus confirmed the CGH indication that a small segment of 9p remained intact in the abnormal chromosome.

A case of AML with chromosome Y monosomy

CGH was carried out on DNA samples from a case of AML which was reported as having a 45X, -Y chromosome constitution. Control DNA was used from a normal male individual. CGH showed loss of DNA material corresponding to the whole chromosome Y. Detailed analysis of the CGH images however detected green fluorescence signals corresponding to the Y chromosome indicating the presence of possible mosaicism or (less likely) insertion of Y material in another chromosome, although the presence of a cell line with an XY sex chromosome complement could not be confirmed using a Y probe due to insufficient material.

A case of CML with 9q-

In one remaining case which was reported as having a normal karyotype, CGH detected a loss of DNA material corresponding to chromosome 9q. This finding could not be confirmed by FISH, due to insufficient material.

Table 3. 8 CGH and banding results in 14 Patients with haematological malignancies

Case No	Sample	Volume	G banding Results	CGH Results	Diagnosis
965046	Bone marrow	<0.5 ml	46,XX, add(9)(p13)inc [4]/ 46,XX[3]	9p-, 9q +	ALL
964615	Bone marrow	0.5-1 ml	46,XX	NAD	MDS
964698	Bone marrow	1-2 ml	46,XY	NAD	AML
965070	Blood	0.5-1 ml	46,XY, del(7)(q34), -17, +r[8]	-17q	AML
965071	Blood	0.5-1 ml	46,XY[17]/47, +8[2]	+8	AML
965108	Blood	<0.5 ml	46,XX	NAD	AML
965469	Bone marrow	<0.5 ml	46,XY, t(9;22)(q34;q11)	NAD	CML
964563	Blood	<0.5 ml	47,XY, +12 [1]	+12	CLL
965140	Bone marrow	0.5-1 ml	45,X,-Y, add (10)(p11.2) del (11)q21), add (12)(p11), add	-Y	AML
965210	Blood	0.5-1 ml	46,XY,[1]	9q-	CML?
965301	Bone marrow	<0.5 ml	46,XY,add(2)(q23 31),t(9;1l)(p22;q23) (9)	NAD	AML
970031	Bone marrow	0.5-1 ml	46,XX, t(9;22)(q34;q11)	NAD	CML
970032	Bone marrow	<0.5 ml	46,XX	NAD	AML
970795	Bone marrow	<0.5 ml	46,XX	NAD	ALL

NAD=No abnormality detected

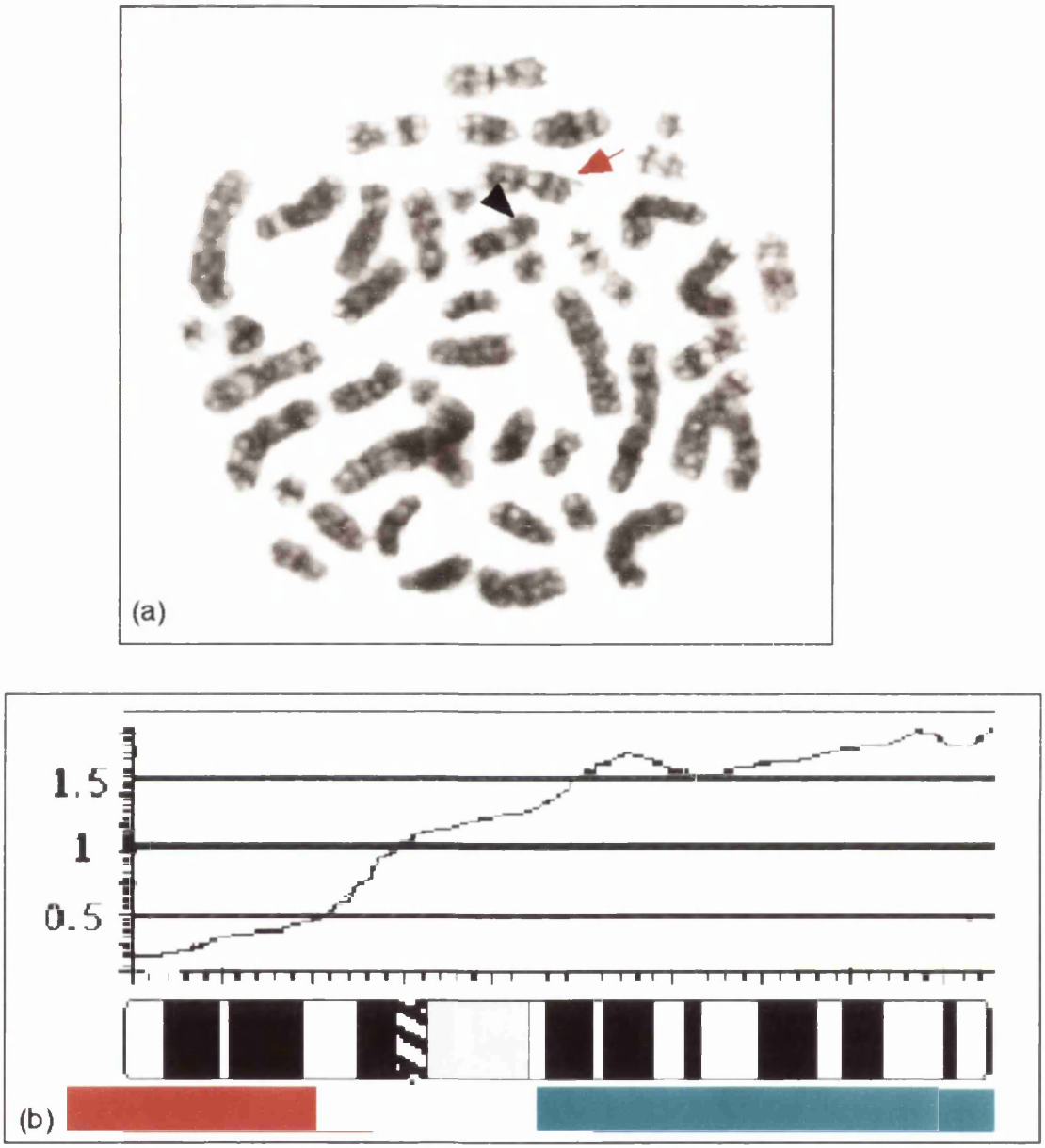


Figure 3.27: (a) G-banded metaphase chromosomes from the case described above. The abnormal and normal chromosome 9s are indicated by red and black arrows respectively. (b) Green (test) to red (control) fluorescence intensity ratio profile of chromosome 9 demonstrating a loss and gain of DNA material corresponding to 9p and 9q respectively. The mean ratio of 20 measurements is shown.

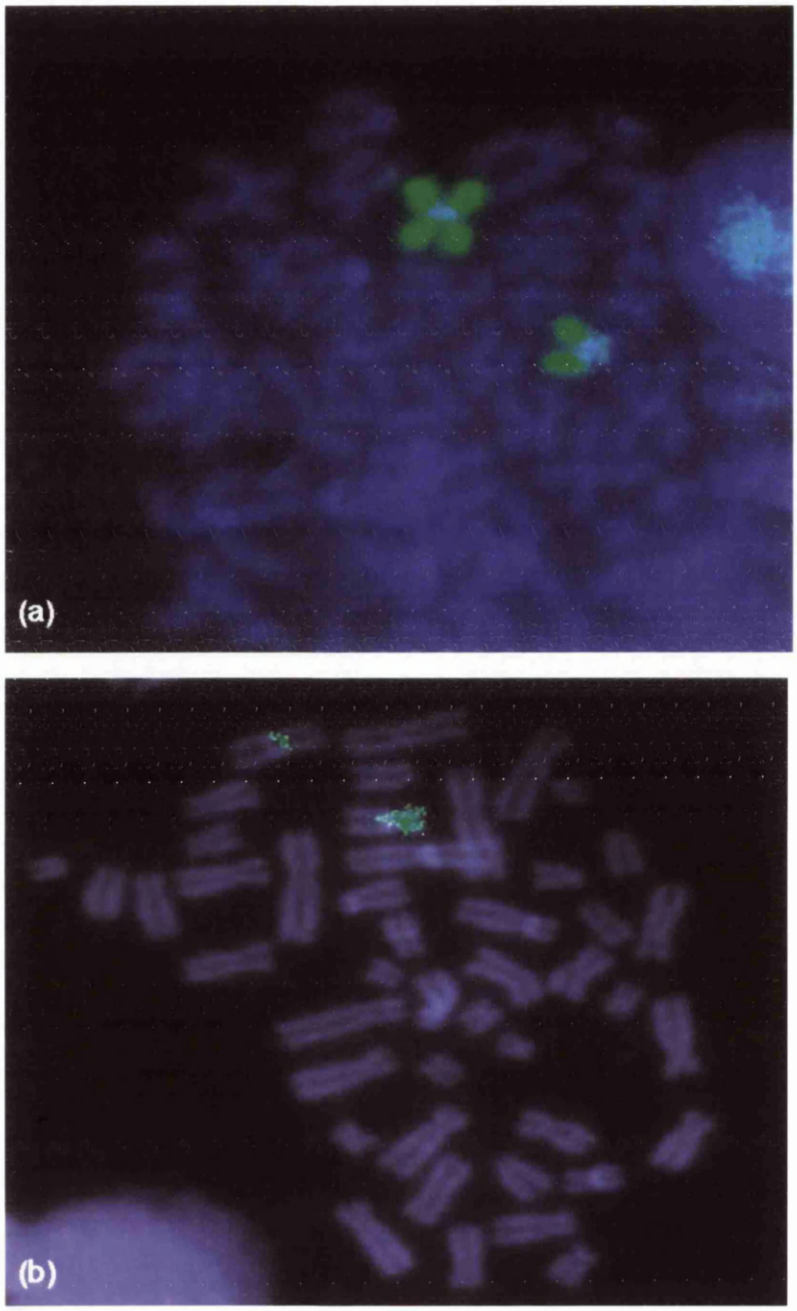


Figure 3. 28: FISH to directly prepared metaphase spreads from the the case described above. (a) Chromosome 9q arm specific painting probe is hybridised to the q arm of the normal chromosome 9 and both arms of the abnormal chromosome 9 confirming the q origin of both arms of the abnormal chromosome 9. (b) Chromosome 9p painting probe is hybridised to the short arm of the normal chromosome 9 and a very small segment of the shorter arm of the abnormal chromosome 9.

3.7 CGH using universally amplified DNA samples

Small amounts of DNA sample as low as 1 ng were used as a template for DOP-PCR (see Methods). Universally amplified DNA was then used as test DNA in CGH experiments. All CGH experiments using this approach could reliably detect the numerical abnormalities in 10 cases studied in this manner. DNA extracted from both blood samples and fixed cells were used in this approach.

Table 3. 9: CGH using universally amplified DNA samples: summary of results:

Sample	Patient's karyotype	Source of DNA	Control DNA	Result	Failures	Amount of DNA used for amplification
1	46,XX,+18	Fixed cells	46,XY	+X,-Y, +18	1	20 ng
2	47,XX,+21	Blood	46,XY	+X,-Y, +21	-	50 ng
3	46,XX	Blood	46,XY	+X,-Y	-	10 ng
4	46,XY	Fixed cells	46,XX	+Y,-X	1	1 ng
5	46,XY,+21	Blood	46,XY	+Y,-X, +21	1	1 ng
6	46,XY,+18	Fixed cells	46,XX	+Y,-X,+18	-	20 ng
7	46,XX	Blood	46,XY	+X,-Y	-	5 ng
8	47,XXX	Blood	46,XX	+X	-	5 ng
9	47,XY,+13	Fixed cells	46,XX	+Y,-X,+13	1	1 ng
10	47,XXY	Fixed cells	46,XY	+X	-	2 ng

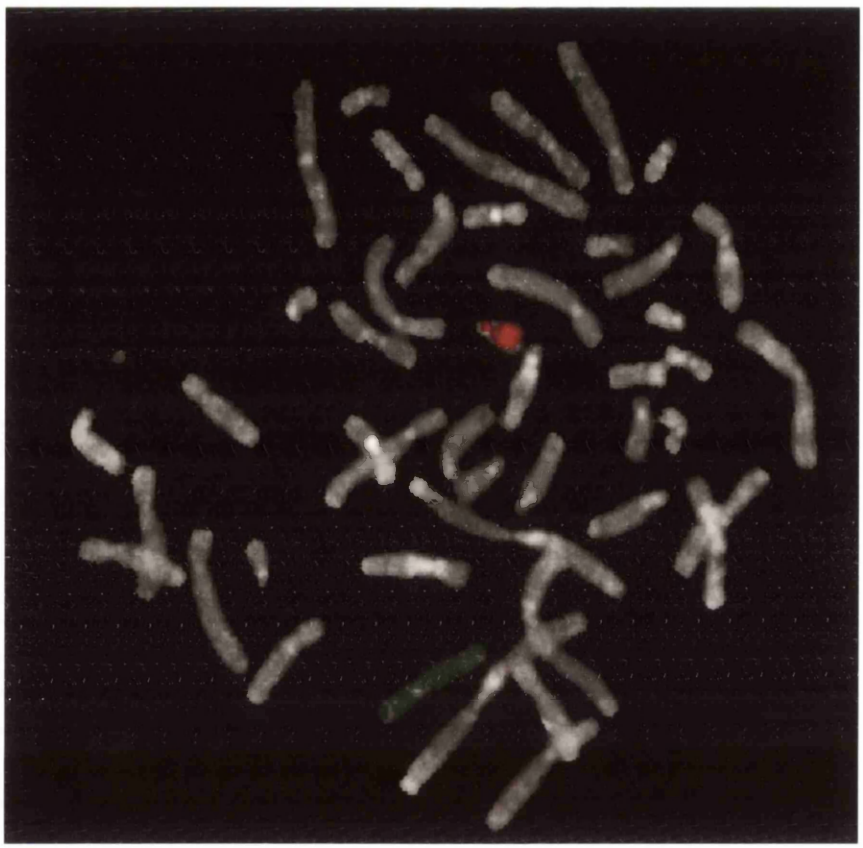


Figure 3. 29: Global CGH results of universally DOP-PCR amplified DNAs from a normal male (red) and a normal female (greend) individual. Chromosome Y and X appear as red and green respectively confirming the copy number differences of these chromosomes between the two DNA samples.

3.8 DNA molecules as hybridisation targets

In this part of the study, the possibility of using DNA molecules as the hybridisation targets to improve the CGH technique resolution was investigated. First, low-density DNA fibres combed on glass slides were used as the targets for comparative cosmid hybridisations. Then high concentration arrays of DNA fibres were the target of comparative genomic hybridisation. Finally, DNA microarrays of ~300-700 bp were targeted.

3.8.1 DNA fibres

Single DNA fibres

Cosmid MA2B3 which contains 35 kb of human dystrophin gene including exon 48 was used as the hybridisation target. MA2B3 was first treated with *Not* I, resulting in linearisation of the cosmid with the vector at one end of the fibre. The 35-kb fragments were then attached to glass slides pre-treated with silane using DNA combing approach (see Methods). Cosmid DNA was then directly labelled with FITC (green) and TR (red). Differentially labelled cosmid DNAs in different ratios were then hybridised to their respective combed cosmid fibre. The chosen ratios of FITC to TR labelled DNA fragments in the hybridisation solution were 1:2, 2:3, 1:1, 3:2, 2:1 and 3:1. Green and red dots along the combed fibres corresponding to FITC and TR labelled fragments were then visualised using a fluorescence microscope and image analysis software (see Methods). Images of 14-22 DNA fibres were acquired by a CCD camera using FITC- and TR-specific filters. For each ratio of FITC and TR-labelled cosmid DNAs the measured fluorescence values for FITC and TR and the FITC to TR ratio was calculated for each DNA fibre. The relative increase of FITC- versus TR-labelled DNA fragments in the probe mixture resulted in an increase of the measured FITC fluorescence in proportion to the TR fluorescence values. (Figure 3. 30 and Table 3. 10 to Table 3. 15)

Table 3. 10: Values corresponding to comparative cosmid hybridisation to 22 DNA fibre targets [FITC (Green)/TexasRed (Red): 2/1

Fibre. no.	DNA fibres (obsereved values)		Background values		Normalised values (observed-background)		Area	Green Mean	Red Mean	Green/Red
	Green Sum	Red Sum	Green Sum	Red Sum	Green Sum	Red Sum				
1	71100	30265	3060	1020	68040	29245	340	200.11	86.01	2.32
2	59197	35339	604	1510	58593	33829	302	194.01	112.01	1.73
3	40115	21635	2310	1260	37805	20375	210	180.02	97.02	1.85
4	81431	40387	1986	662	79445	39725	331	240.015	120.01	1.99
5	56624	28193	5103	1701	51521	26492	243	212.02	109.02	1.94
6	29255	26681	3042	1638	26213	25043	234	112.02	107.02	1.04
7	46821	24249	3135	1672	43686	22577	209	209.02	108.02	1.93
8	65917	31421	3388	1232	62529	30189	308	203.01	98.01	2.07
9	29160	27690	1225	1715	27935	25975	245	114.02	106.02	1.07
10	123089	60845	3744	4212	119345	56633	468	255.01	121.01	2.10
11	32810	14450	405	1080	32405	13370	135	240.03	99.03	2.42
12	35953	21505	1892	2064	34061	19441	172	198.02	113.02	1.75

Table: Cont.

Fibre. No.	DNA fibres (obsereved values)		Background values		Normalised values (observed-background)		Area	Green Mean	Red Mean	Green/Red
	Green Sum	Red Sum	Green Sum	Red Sum	Green Sum	Red Sum				
13	52579	26292	1897	1084	50682	25208	271	187.01	93.018	2.01
14	43364	24740	1746	2328	41618	22412	291	143.01	77.017	1.85
15	27725	14117	1008	1134	26717	12983	126	212.04	103.04	2.05
16	29059	13338	2388	398	26671	12940	199	134.02	65.02	2.06
17	41994	21949	2110	1055	39884	20894	211	189.02	99.02	1.90
18	57293	26789	4092	5580	53201	21209	372	143.01	57.01	2.50
19	50237	29261	1656	1932	48581	27329	276	176.01	99.01	1.77
20	74436	43909	4116	3087	70320	40822	343	205.01	119.01	1.72
21	55557	28432	2821	2387	52736	26045	217	243.02	120.02	2.02
22	56723	27679	1096	822	55627	26857	274	203.01	98.01	2.07

Table 3. 11: Values corresponding to comparative cosmid hybridisation to 17 DNA fibre targets [FITC (Green)/TR (Red): 3/1]

Fibre. no.	DNA fibres (obsereved values)		Background values		Normalised values (observed-background)		Area	Green Mean	Red Mean	Green/Red
	Green Sum	Red Sum	Green Sum	Red Sum	Green Sum	Red Sum				
1	55815	19920	668	1659	55147	18261	280	196.95	65.22	3.02
2	66336	22038	3369	1120	62967	20918	310	203.12	67.48	3.01
3	79594	26882	2188	726	77406	26156	318	243.42	82.25	2.96
4	68017	22741	3730	1353	64286	21388	312	206.05	68.55	3.01
5	52790	17911	2090	1190	50700	16721	267	189.89	62.63	3.03
6	44540	16134	1924	2559	42616	13575	293	145.45	46.33	3.14
7	49218	16875	1209	902	48009	15972	233	206.05	68.55	3.01
8	79880	30358	4122	4631	75758	25727	293	258.56	87.81	2.94
9	39874	14809	2085	2268	37790	12540	188	201.01	66.7	3.01
10	64960	22985	449	1186	64511	21799	265	243.44	82.26	2.96
11	68242	23676	1112	1245	67130	22430	312	215.16	71.89	2.99

Table: Cont.

Exp. no.	DNA fibres (obsereved values)		Background values		Normalised values (observed-background)		Area	Green Mean	Red Mean	Green/Red
	Green Sum	Red Sum	Green Sum	Red Sum	Green Sum	Red Sum				
12	28347	9432	3350	1800	24997	7633	219	114.14	34.85	3.28
13	34689	12099	1351	1885	33338	10215	287	116.16	35.59	3.26
14	62629	20919	5617	1869	57012	19050	265	215.14	71.88	2.99
15	38767	11831	2630	436	36137	11395	265	136.37	43	3.17
16	83204	29718	3106	2624	80097	27094	325	246.45	83.37	2.96
17	42034	14352	2544	1384	39490	12968	216	182.82	60.04	3.05

Table 3. 12: Values corresponding to comparative cosmid hybridisation to 17 DNA fibre targets [FITC (Green)/TR (Red): 3/2]

Fibre. no.	DNA fibres (obsereved values)		Background values		Normalised values (observed-background)		Area	Green Mean	Red Mean	Green/Red
	Green Sum	Red Sum	Green Sum	Red Sum	Green Sum	Red Sum				
1	76358	56588	1723	8357	74635	48231	349	213.88	138.21	1.55
2	57834	46773	825	3238	57009	43535	275	207.29	158.3	1.31
3	53084	39698	1015	4107	52069	35591	237	219.71	150.18	1.46
4	46592	33883	3407	1431	43184	32452	213	202.78	152.38	1.33
5	100948	69331	421	5049	100527	64282	421	238.92	152.78	1.56
6	63547	43602	3709	1192	59839	42411	298	200.82	142.33	1.41
7	90861	57337	3540	3704	87321	53633	405	215.54	132.39	1.63
8	68474	45924	2415	2267	66059	43657	321	205.86	136.05	1.51
9	42132	28692	1764	2749	40368	25943	183	220.66	141.81	1.56
10	64043	47159	130	5673	63913	41486	295	216.55	140.56	1.54

Table: Cont.

Exp. no.	DNA fibres (obsereved values)		Background values		Normalised values (observed-background)		Area	Green Mean	Red Mean	Green/Red
	Green Sum	Red Sum	Green Sum	Red Sum	Green Sum	Red Sum				
11	81606	59336	3608	2409	77998	56927	374	208.74	152.35	1.37
12	89014	67641	1576	3849	87437	63791	415	210.61	153.65	1.37
13	58927	40051	1725	4000	57202	36050	275	207.9	131.03	1.59
14	48502	28270	2496	2180	46006	26090	227	202.73	114.97	1.76
15	46550	39215	1006	1688	45543	37526	243	187.18	154.23	1.21
16	40602	25487	3414	1549	37188	23938	176	210.87	135.74	1.55

Table 3. 13: Values corresponding to comparative cosmid hybridisation to 14 DNA fibre targets [FITC (Green)/TR (Red): 1/1]

Fibre. no.	DNA fibres (obsereved values)		Background values		Normalised values (observed-background)		Area	Green Mean	Red Mean	Green/Red
	Green Sum	Red Sum	Green Sum	Red Sum	Green Sum	Red Sum				
1	41675	58449	232	1497	41444	56952	232	179	245.98	0.72
2	85895	77561	1078	719	84816	76842	359	235.98	213.8	1.10
3	101374	89091	3824	1561	97550	87530	383	255	228.81	1.11
4	84110	86966	1640	675	82469	86291	338	244.31	255.64	0.95
5	54340	51954	3099	3412	51241	48542	219	233.5	221.2	1.05
6	65085	69967	283	2206	64802	67761	275	235.23	245.97	0.95
7	106865	105170	5708	7953	101157	97217	415	243.66	234.17	1.04
8	65895	65567	4707	2132	61188	63435	254	240.91	249.76	0.96
9	72739	70076	3080	1732	69659	68344	289	241.32	236.76	1.01
10	62811	81090	2443	5494	60369	75596	318	190	237.93	0.79

Table: Cont.

Exp. no.	DNA fibres (obsereved values)		Background values		Normalised values (observed-background)		Area	Green Mean	Red Mean	Green/Red
	Green Sum	Red Sum	Green Sum	Red Sum	Green Sum	Red Sum				
11	79595	78927	356	1871	79239	77056	317	249.69	242.81	1.028
12	71268	60545	5790	1304	65478	59241	261	251.09	227.18	1.10
13	81881	82601	896	2951	80985	79650	321	252	247.85	1.01
14	73008	103052	1694	969	71314	102083	432	165	236.19	0.69

Table 3. 14: Values corresponding to comparative cosmid hybridisation to 18 DNA fibre targets [FITC (Green)/TR (Red): 2/3]

Fibre. no.	DNA fibres (obsereved values)		Background values		Normalised values (observed-background)		Area	Green Mean	Red Mean	Green/Red
	Green Sum	Red Sum	Green Sum	Red Sum	Green Sum	Red Sum				
1	47098	86723	2392	1008	44706	85715	336	133	255	0.52
2	54597	90103	4753	4070	49845	86034	348	143.1	247	0.58
3	68578	86253	6487	3731	62091	82522	382	162.61	216.11	0.75
4	51052	95584	3502	3572	47550	92011	361	131.66	254.76	0.52
5	33668	51429	1417	3862	32251	47567	215	149.85	221.01	0.68
6	30503	40561	3042	2840	27461	37721	272	101.09	138.86	0.73
7	42210	65963	3702	2534	38507	63429	294	131.13	216	0.61
8	47628	60475	1304	556	46325	59918	314	147.4	190.66	0.77
9	54743	64899	5944	1007	48799	63891	317	154	201.63	0.76
10	59489	44582	6766	3297	52723	41285	328	160.96	126.04	1.28

Table : Cont.

Fibre. no.	DNA fibres (obsereved values)		Background values		Normalised values (observed-background)		Area	Green Mean	Red Mean	Green/Red
	Green Sum	Red Sum	Green Sum	Red Sum	Green Sum	Red Sum				
11	50662	94162	3602	678	47059	93484	367	128.37	255	0.5
12	55797	68798	4905	934	50892	67864	319	159.73	213	0.75
13	31134	60728	1277	474	29857	60254	237	125.86	254	0.5
14	55538	97297	2059	2963	53479	94334	371	143.99	254	0.57
15	30025	37491	1778	2173	28246	35318	181	155.74	194.73	0.8
16	43459	75376	2615	445	40845	74931	294	139	255	0.55
17	29938	29927	583	199	29355	29727	199	147.36	149.23	0.99
18	64976	85005	6707	343	58269	84662	343	170	247	0.69

Table 3. 15: Values corresponding to comparative cosmid hybridisation to 19 DNA fibre targets [FITC (Green)/TR (Red): 1/2]

Fibre. no.	DNA fibres (obsereved values)		Background values		Normalised values (observed-background)		Area	Green Mean	Red Mean	Green/Red
	Green Sum	Red Sum	Green Sum	Red Sum	Green Sum	Red Sum				
1	32031	78474	2804	6318	29226	72156	329	88.72	219.04	0.41
2	44609	67350	4726	323	39883	67027	422	94.53	158.87	0.6
3	35524	69433	5117	4166	30407	65266	268	113.56	243.74	0.47
4	41350	75807	2544	1147	38806	74660	352	110.23	212.08	0.52
5	40970	38783	7220	3205	33750	35579	323	104.5	110.16	0.95
6	33832	37857	2103	3313	31729	34544	260	122.25	133.09	0.92
7	14127	22850	778	400	13349	22450	124	107.61	180.98	0.59
8	40069	71399	4675	2446	35394	68952	346	102.3	199.29	0.51
9	39529	90619	2084	7204	37445	83415	349	107.35	239.15	0.45
10	23601	59397	3069	1289	20532	58108	277	74.02	209.48	0.35

Table: Cont.

Fibre. no.	DNA fibres (obsereved values)		Background values		Normalised values (observed-background)		Area	Green Mean	Red Mean	Green/Red
	Green Sum	Red Sum	Green Sum	Red Sum	Green Sum	Red Sum				
11	21404	38845	3425	2839	17979	36006	197	91.07	182.38	0.5
12	55863	104951	11488	1266	44375	103685	444	99.92	233.46	0.43
13	40550	59536	6002	1665	34548	57871	329	105.12	176.09	0.6
14	51033	114365	7094	3399	43939	110966	395	111.18	280.77	0.4
15	52460	70146	2562	3110	49898	67036	435	114.84	154.28	0.74
16	36407	87978	2305	4040	34102	83938	398	85.61	210.73	0.41
17	56430	54455	9855	54	46575	54401	392	118.91	138.9	0.86
18	35475	72536	5309	1927	30165	70609	365	82.65	193.45	0.43
19	37671	66954	4262	1938	33410	65016	306	109.25	212.6	0.51

Table 3. 16: Comparative cosmid hybridisation to 106 DNA fibres: summary of results

Ratio of FITC/TexasRed labelled DNA in hybridisation solution	No. fibres examined	Mean of observed Green/red
3/1	17	3.05
2/1	22	1.92
3/2	16	1.48
1/1	14	0.97
2/3	18	0.69
1/2	19	0.55

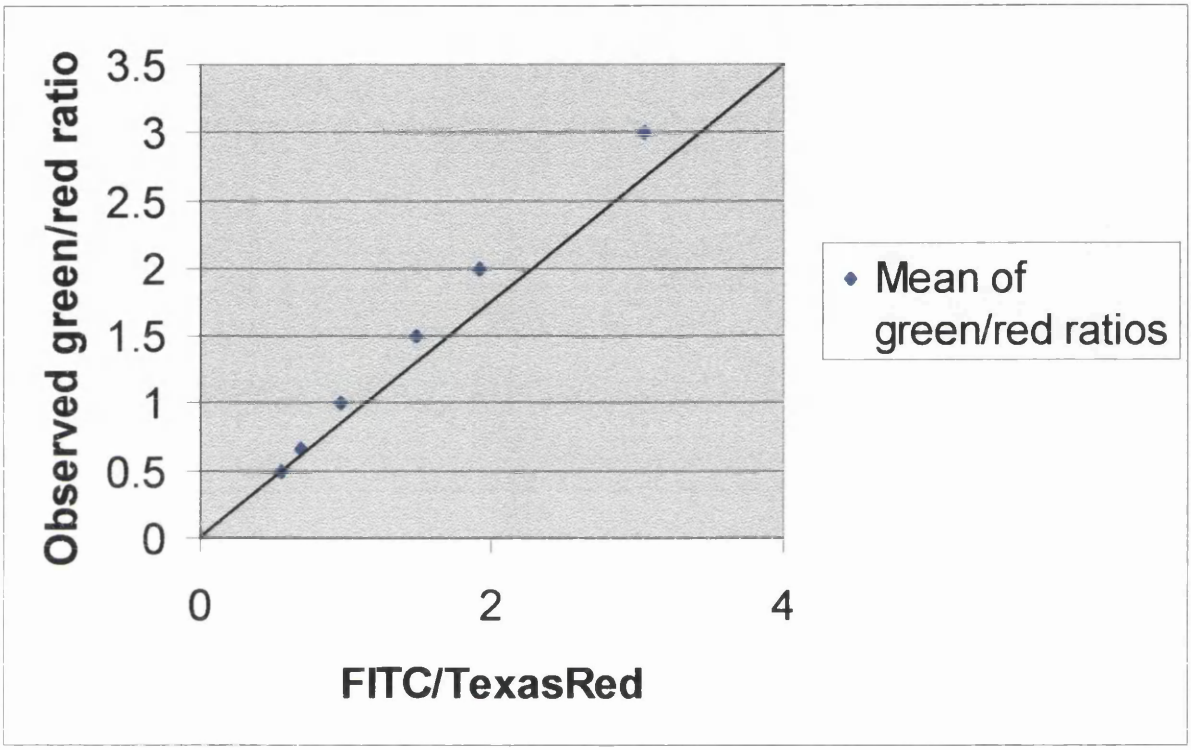


Figure 3. 30: Mean of normalised green/red ratios plotted against the fluorochrome ratios

CGH to dotted high density DNA fibres

In this part of the study, high-density DNA fibres were used as hybridisation targets (Figure 3. 31). <0.1 to 0.3 μl of solutions containing ~100-300 ng of cosmid DNA were spotted on silane or poly-l-lysine pre-treated glass slides (see Methods, Figure 3. 32 and Figure 3. 33). Differentially labelled genomic DNAs from normal male and females were then co- hybridised to the DNA spots. Normalised green to red ratios of measured fluorescence intensities were directly related to the relative copies of chromosomes X and Y in the differentially labelled DNAs.

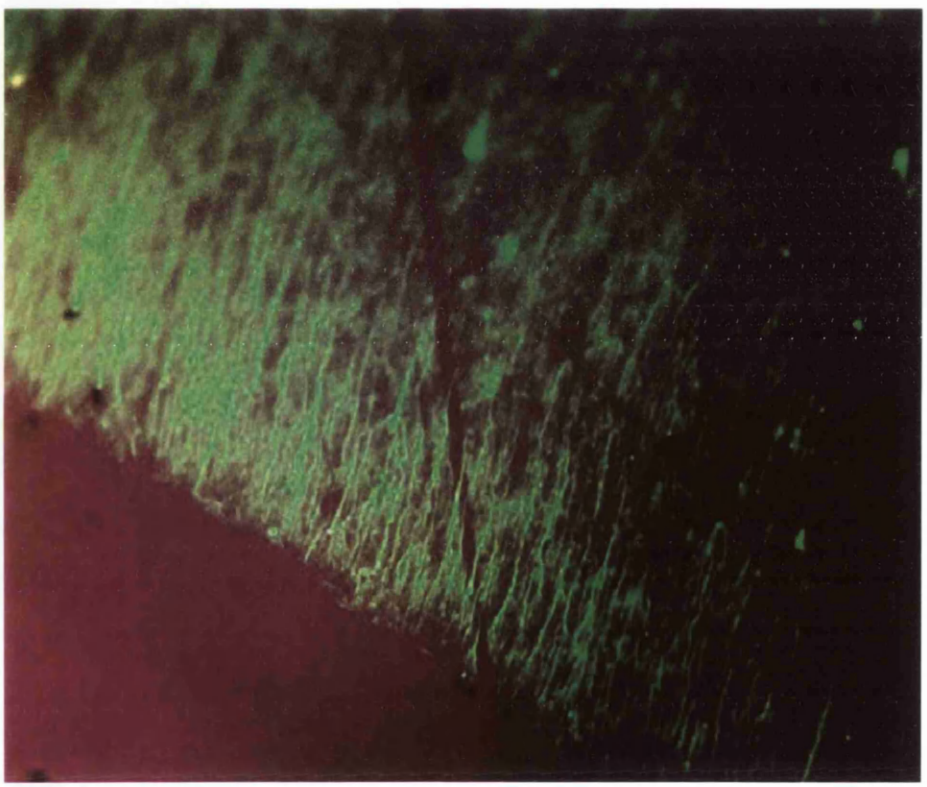


Figure 3. 31: 400x magnification of YOYO-1 stained high-density cosmid fibres containing exon-48 of human *DMD* gene.

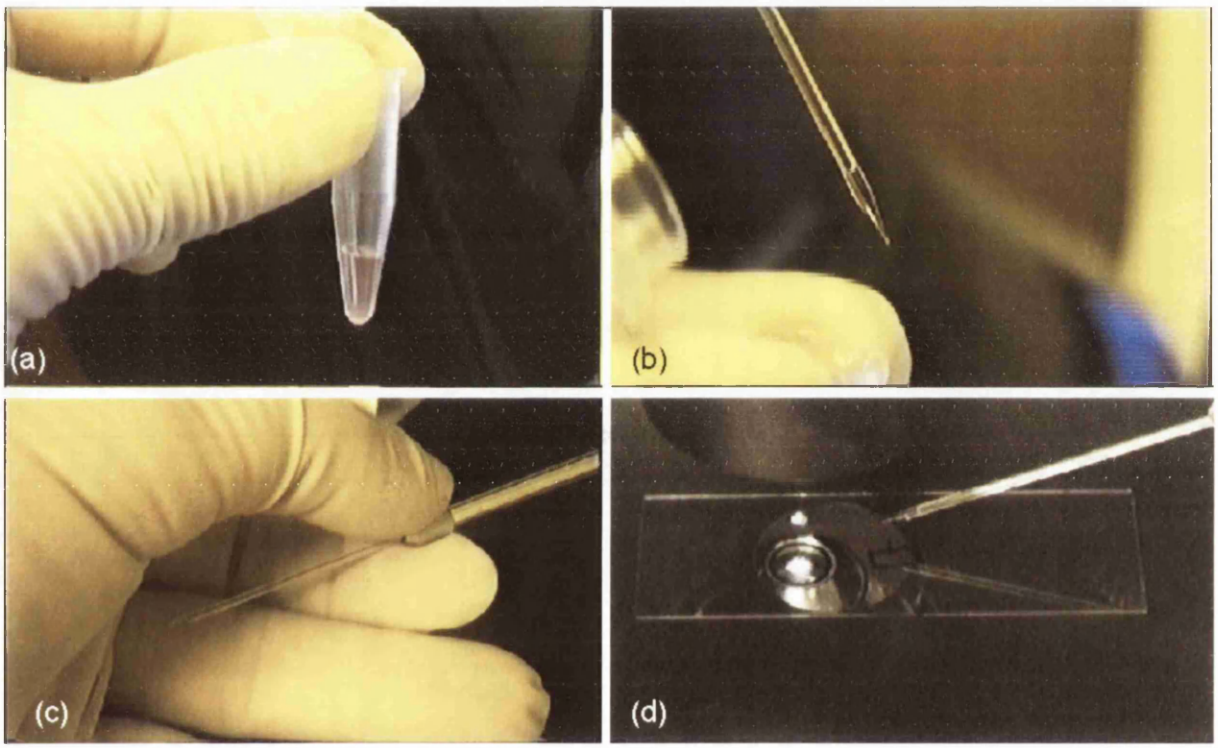


Figure 3. 32: Spotting of DNA targets on pre-treated glass slides for CGH.
(a) A glass needle is put in the DNA solution. (b) The DNA is sucked to the glass needle by capillary effect. (c) The needle containing the target DNA is then assembled on the inverted microscope and (d) the DNA solution is put on the glass slide guided by use of the inverted microscope.

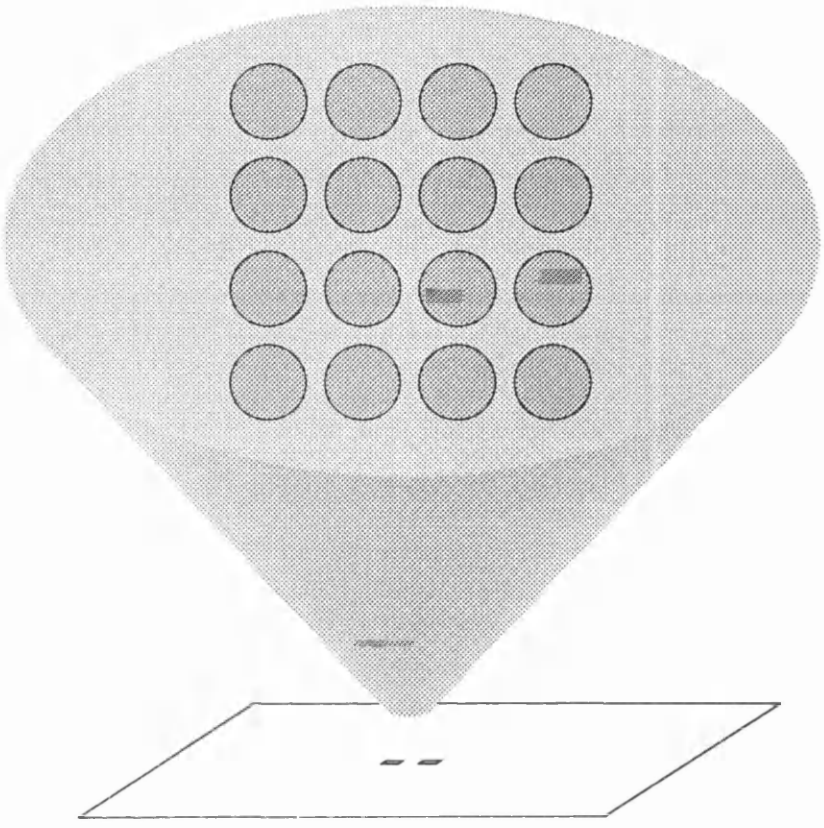


Figure 3. 33: A close up view of the immobilised spotted DNA fragments.

For each hybridisation area, 16 DNA spots were spaced at a distance of 4-10μm (where glass capillaries used under the inverted microscope) to 400μm (where micropipettes used) from centre to centre.

Table 3. 17: CGH to high density DNA fibres dotted on glass slides

Target spot	Group 1 (XX green, XY red) Fluorescence intensity			Group 2 (XX Red, XY green) Fluorescence intensity		
	Green (XX)	Red (XY)	Green/Red (XX/XY)	Green (XY)	Red (XX)	Red/Green (XX/XY)
1	233671	123429	1.89	765919	1457990	1.90
2	3386842	1928879	1.76	2548985	5485995	2.15
3	4315732	2316494	1.86	2335761	5728764	2.45
4	11503311	5175937	2.22	1866147	3340121	1.79
5	1522375	718576	2.12	1501678	2749727	1.83
6	2392204	1421344	1.68	1318542	2603683	1.97
7	6657908	3872820	1.72	5487636	9070159	1.65
8	4710801	2944016	1.60	1090480	2561052	2.35
9	4319301	2500463	1.73	2492877	5409952	2.17
10	4426536	2354130	1.88	138140	283399	2.05
11	2551258	1512706	1.69	1340842	3303440	2.46
12	5961115	3123223	1.91	1379924	3312934	2.40
13	10590421	5453315	1.94	2884739	4961156	1.72
14	2035965	1141460	1.78	3343475	6824490	2.04
15	6726211	3926917	1.71	3425583	6772805	1.98
16	12843629	7072546	1.82	1796527	3457514	1.92

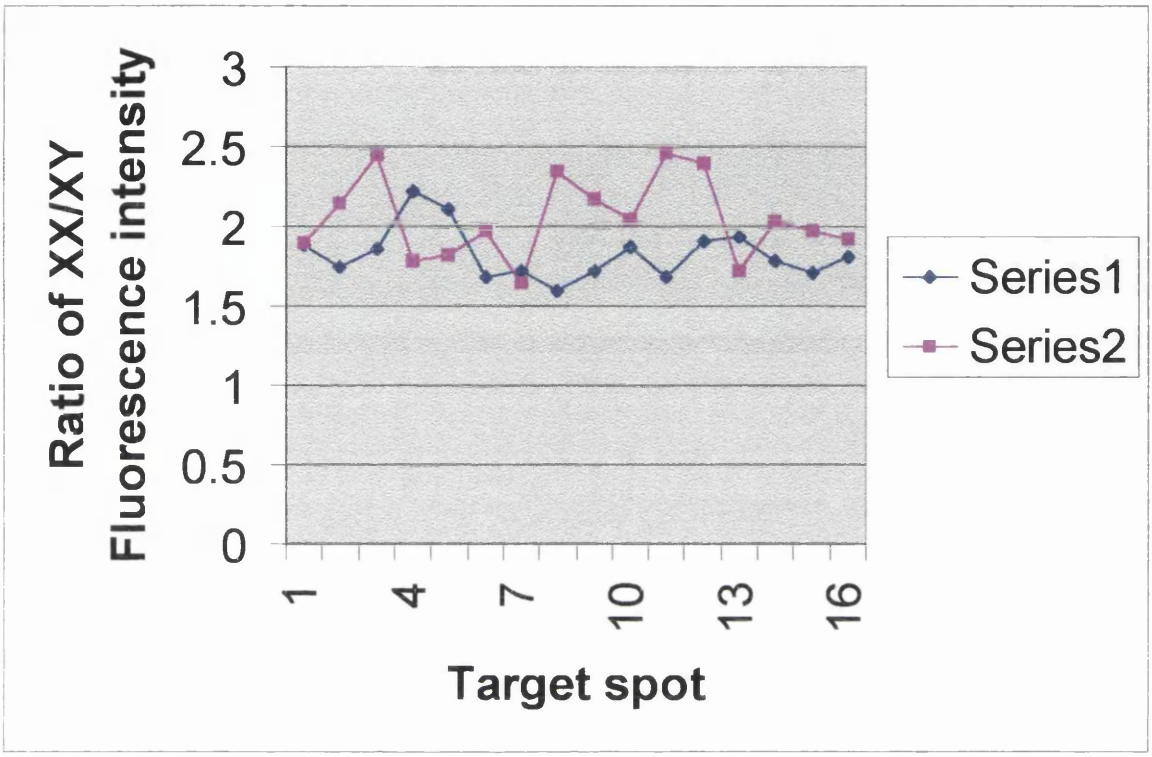


Figure 3. 34: Graphic depiction of results shown in Table 3. 17. Fluorescence intensity ratios in CGH to two groups of 16 DNA spots are shown together. As illustrated XX/XY ratio despite the alteration of the fluorochromes remained almost the same confirming the direct relationship between the copy numbers of X chromosomes and the corresponding fluorochromes. Significance of these findings are discussed in next chapter.

3.8.2 DNA microarrays

Optimisation

Three target sequences, corresponding to the exon 48 of human *DMD* gene on chromosome X, *SRY* gene on chromosome Y and *hMSH2* gene on chromosome 2 were selected. Using appropriate oligonucleotide primers, target DNA segments were amplified (Figure 3. 35). The amplified DNA with the concentration of1 μg/ μl were then stained with YOYO-1 and spotted on silane or poly-l-lysis treated glass slides (see Methods).

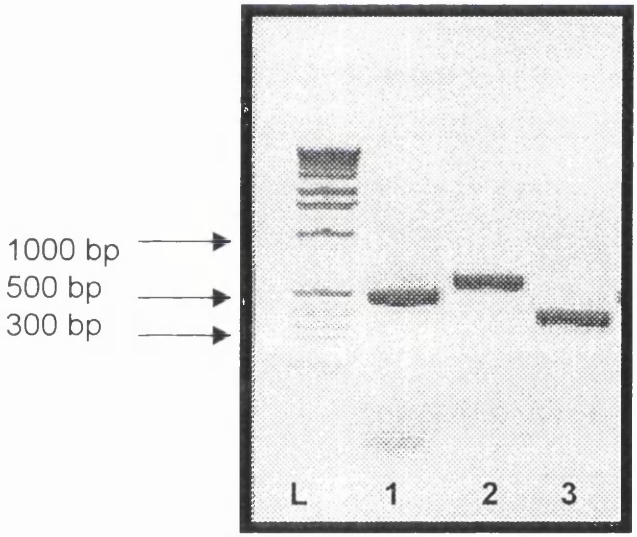


Figure 3. 35: Agarose gel showing amplification products from *SRY* lane (1) *DMD* lane (2) and *hMSH2* genes lane (3). L=1 kb DNA Ladder.

A routine denaturation protocol which involved 70°C formamide approach (see Chapter 2) was carried out. The YOYO-1 stained DNA spots were then checked under a fluorescence microscope. DNA spots were not detectable, indicating that most of the DNA arrays had been washed out during the denaturation and washing procedures and those remaining were insufficient to be visible.

Denaturation of the DNA targets in 100°C distilled water (instead of formamide) for one minute improved the DNA yield but was still not acceptable for fluorescence hybridisation. To bypass the denaturation stage, it was decided to make the DNA targets single stranded before spotting them on the glass slide.

The PCR products were made single stranded using magnetic beads (Figure 3. 36)(see Methods).

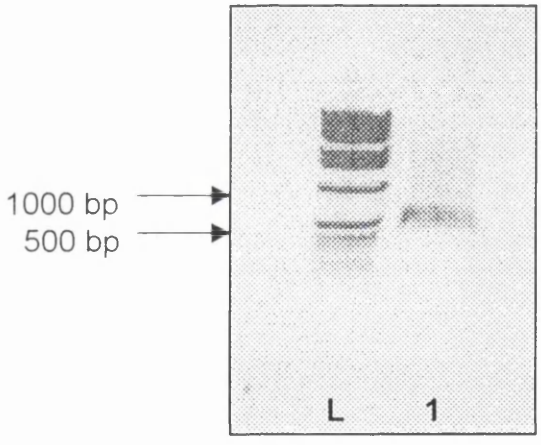


Figure 3. 36: Agarose gel showing single stranded PCR product of *DMD* gene (1). Single stranded DNA is produced using magnetic beads. Details of the procedure are described in chapter 2. L=1 kb DNA Ladder.

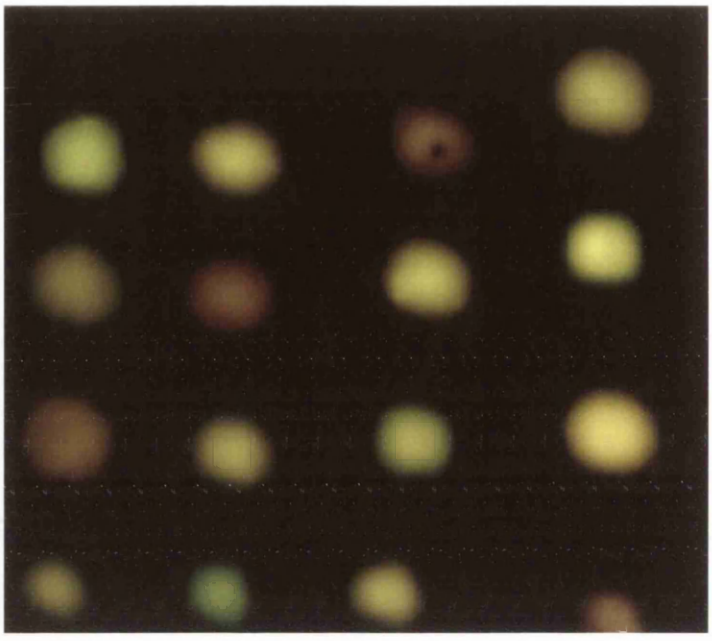
The single stranded DNA products were then spotted on silanised glass alternatively in a manner that the DNA targets corresponding to the X and Y chromosomes were separated by a spot corresponding to chromosome 2 DNA. In each slide, 16 spots were produced (see Methods). Fluorescence intensity corresponding to the YOYO-1 stained spots were then measured before the slides were incubated at 37°C for 48 hours followed by washing steps similar to those of standard CGH. The fluorescence intensities corresponding to the spots were measured again and compared to those obtained before incubation and washing steps. It was found that more than 60% of the DNA had remained on the slides despite incubation and washing steps. Based on this finding it was concluded that the previously single stranded DNA were the best choice as the target of hybridisation.

CGH to Single stranded DNA arrays

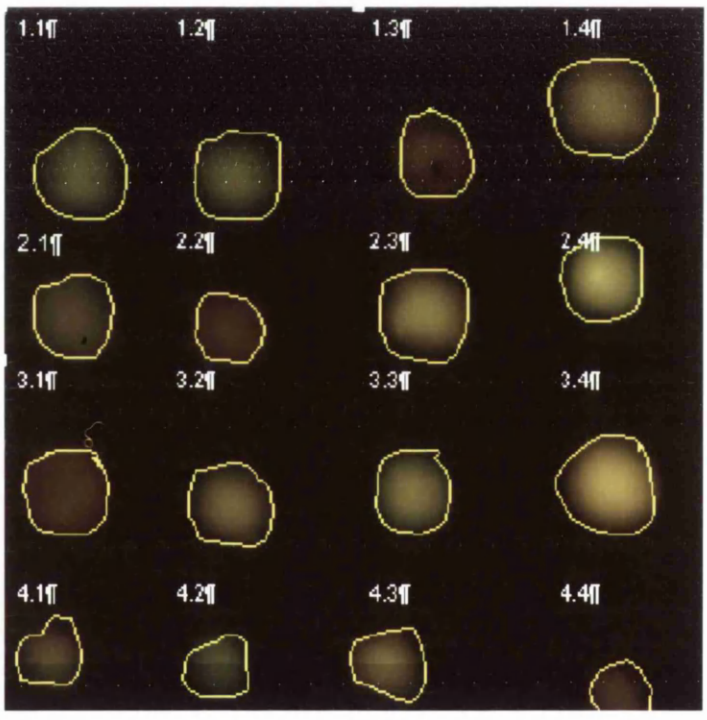
Un-stained single stranded DNA arrays corresponding to the exon 48 of human *DMD* gene, *SRY* gene and *hMSH2* gene were spotted on glass slides. Equal amounts (2-7 μg) of differentially labelled DNAs corresponding to normal male and normal female individuals were co-hybridised to the DNA microarrays. After 48 hours of hybridisation and washing steps, the spots were examined under x10 magnification of fluorescence microscope. Areas of similar sizes were chosen and total fluorescence values for that area were measured.
The ratios of averaged values of red and green signals corresponding to the autosome spots were calculated and were used as correction values for the normalisation of averaged red and green fluorescence values corresponding to *DMD* and *SRY* spots. Finally the corrected fluorescence value for each spot was calculated and the fluorescence ratio of each spot was obtained by dividing the corrected green with the corrected red fluorescence intensity.

No significant difference of the green to red ratio between different spots was detected where less than 7 μg of each DNA had been used.

Where 7 μg of each hybridising DNA (normal male and normal female) was used a significant difference of the fluorescence ratio between *DMD* and *SRY* spots was present. Therefore a hybridisation solution containing 7 μg of each DNA was used for the next stage of the study.



(a)



(b)

Figure 3. 37: CGH to single stranded DNA microarrays (normalised image): (a) Differentially labelled DNAs from a normal female (green) and a normal male (red) are co-hybridised to 16 spots of single stranded DNA microarrays corresponding to *DMD*, *hMSH2* and *SRY* genes. (b) *DMD* and *SRY* spots are positioned at 1.1, 2.4, 3.3, 4.2 and 1.3, 2.2, 3.1, 4.4 respectively. The remaining spots corresponding to the *hMSH2* gene are used as controls. Data extracted from this image is summarised in Table 3. 18.

Table 3. 18: Data extracted from the image shown in Figure 3. 37.

Position	Sequence	Area	Total Green	Total Red	Expected G/R ratio	Observed G/R ratio
1.1	DMD	147	6596	3769	2	1.75
1.2	hMSH2	142	5665	5664	1	1.00
1.3	SRY	156	1007	5304	0	0.19
1.4	hMSH2	172	4562	4905	1	0.93
2.1	hMSH2	159	4545	4573	1	1.00
2.2	SRY	146	1403	4526	0	0.31
2.3	hMSH2	152	4683	5098	1	0.92
2.4	DMD	153	5349	2922	2	1.83
3.1	SRY	155	558	4650	0	0.12
3.2	hMSH2	136	4675	4807	1	0.97
3.3	DMD	148	4503	2602	2	1.73
3.4	hMSH2	164	5123	5568	1	0.92
4.1	hMSH2	114	3758	3660	1	1.03
4.2	DMD	117	4920	2392	2	2.05
4.3	hMSH2	128	5155	5300	1	0.97
4.4	SRY	91	247	3094	0	0.08

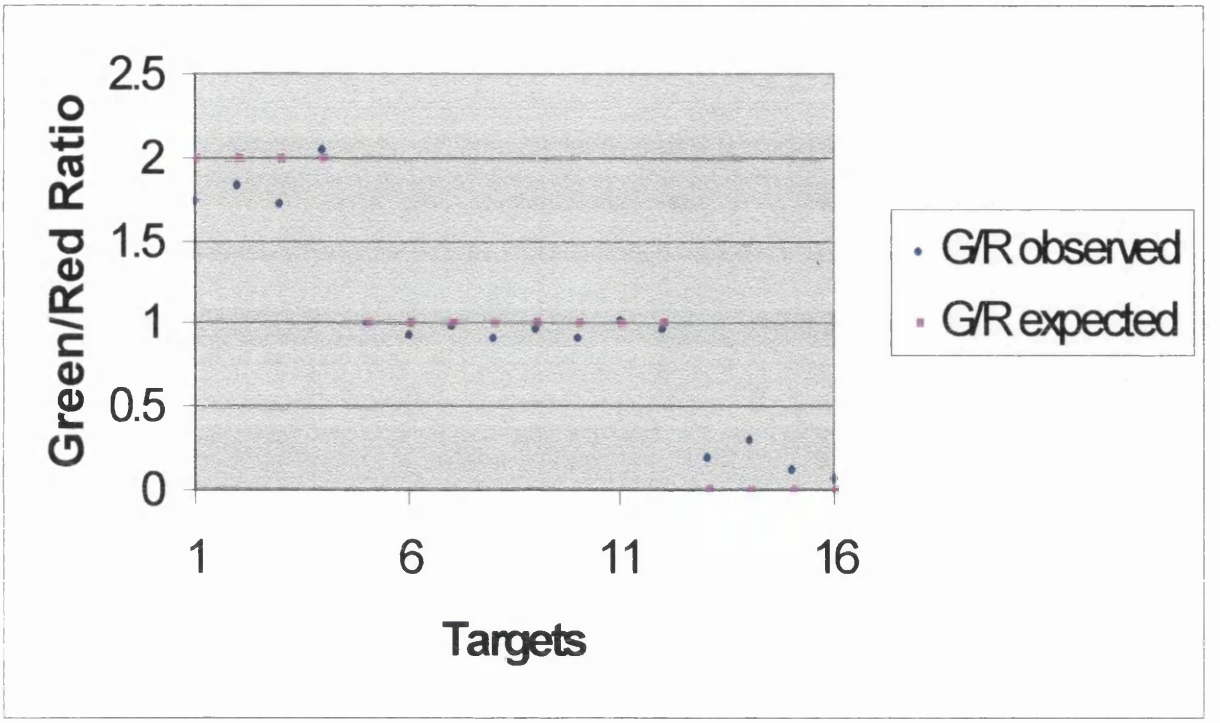


Figure 3. 38 Scatter graphic of the results shown in Table 3. 18. Results corresponding to DMD and SRY spots are shown from 1-4 and 13-16 respectively. Results from the control spots are shown between the two (5-12). As illustrated a significant difference between the Green/Red ratio corresponding to DMD and SRY spots is clear.

DNA samples from 2 male individuals with complete deletion of exon 48 of *DMD* gene were then used as test DNA in CGH experiments to DNA array targets made of exon 48 of *DMD* gene. SRY targets were used as controls. Differentially labelled (TexasRed) DNA samples from normal male individuals were used as control in the hybridisation mixture. Using the same approach described earlier, green to red fluorescence ratios corresponding to the test and to the control samples were calculated. A linear relationship between the observed ratio and the expected values for deletion was found. Results are summarised in Table 3. 19.

Table 3. 19: Data from CGH to 2 groups of 9 spots of single stranded DNA microarrays corresponding to *DMD and SRY* genes using DNA from two patients with complete deletion of exon 48 of *DMD*. DNA from a normal male individual was used as normal control (red).

Position	Sequence	Patient 1 (green) v Normal male (red)		Patient 2 (green) v Normal male (red)	
		Expected G/R ratio	Observed G/R ratio	Expected G/R ratio	Observed G/R ratio
1.1	DMD	0	0.3	0	0.08
1.2	SRY	1	1.11	1	1.05
1.3	DMD	0	0.11	0	0.17
2.1	SRY	0	1.00	1	0.99
2.2	DMD	1	0.08	0	0.34
2.3	SRY	1	0.99	1	0.93
3.1	DMD	0	0.12	0	0.07
3.2	SRY	1	1.02	1	0.97
3.3	DMD	0	0.02	0	0.11

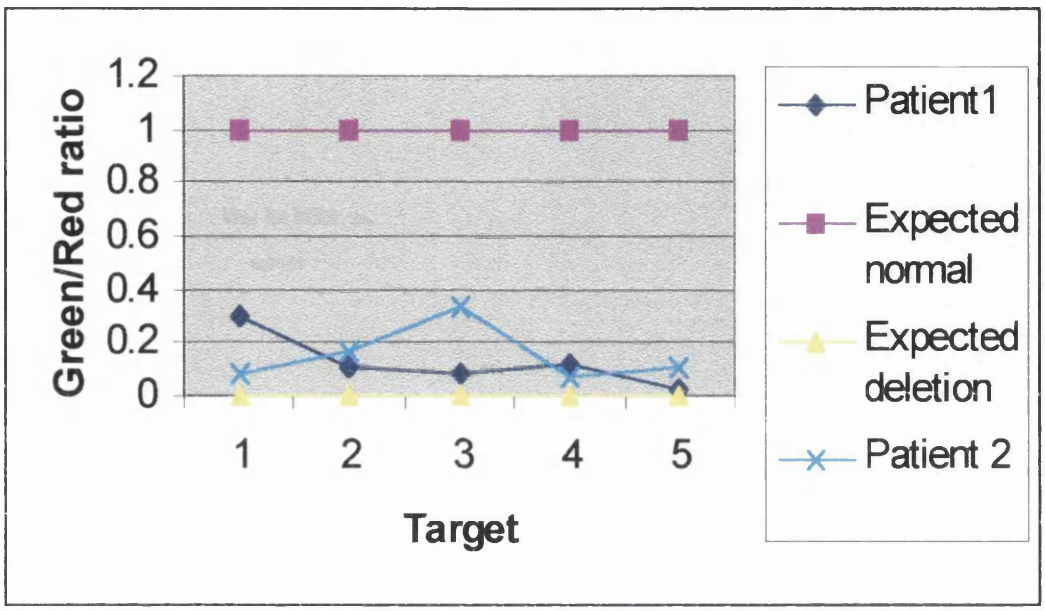


Figure 3. 39: Graphic depiction of the results shown in Table 3. 19. Results corresponding to 9 DMD spots from 2 experiments are shown. As illustrated the observed ratio values from both patients are significantly shifted towards the expected value for the deletion of the exon 48 of the *DMD* gene.

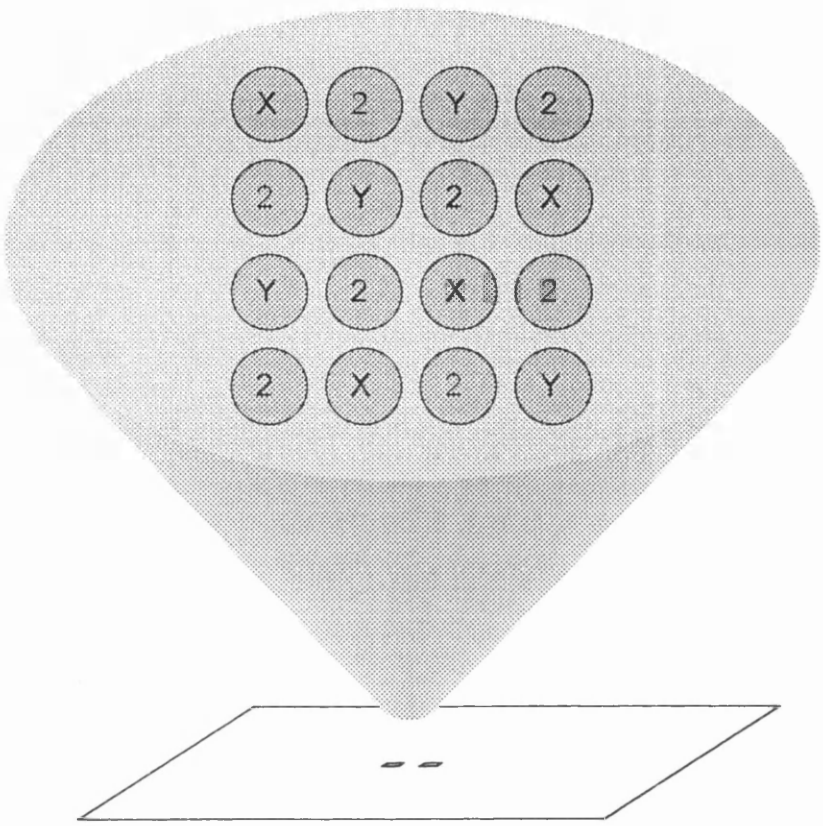


Figure 3. 40: A close up view of the immobilised spotted DNA fragments. For each hybridisation area, 16 DNA spots were spaced at a distance of 4-10μm (where glass capillaries used under the inverted microscope) to 400μm (where micropipettes used) from centre to centre.

4:Discussion

4.1 Optimisation of the CGH technique for clinical cytogenetics laboratory

The initial part of this work was to optimise the CGH method for use in clinical cytogenetics. Standard methods at the time of the beginning of this study were first used (Kallioniemi et al 1992; du Manoir et al 1993). Three experiments performed using the direct method and four experiments using the indirect method failed to produce analysable results.

The modified approach described in this study reduced the time required for the experiment in various steps of the CGH experiment. Meanwhile the technique proved more reproducible and reliable.

4.1.1 Target metaphase

It is now generally accepted that metaphase preparations ideal for G-banding analysis are not necessarily optimal for CGH analysis. Earlier studies had recommended some measures for evaluating the target metaphases for CGH. One (Kallioniemi et al 1994) suggested preparing a large numbers of slides from one batch and carrying out a CGH experiment on several slides from that particular batch. If the results were acceptable then the remaining slides from that particular batch would be used, otherwise they would be abandoned. Another suggestion (Lichter et al 1995) was to evaluate each particular batch of slides by performing mono-colour genomic hybridisation.

The finding of this study that the quality of hybridisation could be predicted by DAPI counterstaining after denaturation of the target metaphases, has an important impact on reducing the time and expense of the metaphase evaluation.

Proteinase K treatment of the target metaphases was not found to have a major impact on the quality of the hybridisation and was therefore abandoned from the protocol.

4.1.2 Denaturation conditions

Increasing the denaturation temperature or time improves the intensity of hybridisation signals but at the same time damages the chromosome morphology. As shown in Figure 3. 1 the best results were achieved when the slides were incubated at 74°C for 3 minutes. While this condition is much harsher than that in FISH, it is very close to that recommended by Kallioniemi et al (1992-1994). However, it should be mentioned that these parameters may require to be altered for some batches of slides.

4.1.3 Genomic DNA concentration

In the modified approach the amount of the test and control DNAs used for the hybridisation has been increased up to 1 μg. In standard protocols reported up to the time of beginning this study (1995), 200-400 ng of DNA was used for each hybridisation. Increasing the amount of the genomic DNA used for the hybridisation up to 1 μg was found to increase the quality of hybridisation. As shown in overnight CGH, using up to 5 μg of each genomic DNA has no adverse effect on the hybridisation.

4.1.4 Genomic DNA labelling

The main advantage of indirect over direct labelling of DNA probes is the strong intensity of hybridisation signals obtained by indirect labelling after several layers of signal amplification. However, direct labelling using higher concentrations of hybridising DNA yields hybridisation signals with strong intensity, low background and smooth coverage of the target chromosomes which are the requirements for CGH. Moreover CGH protocols which use directly labelled genomic DNA are simpler and require a shorter time.

Early reports mentioned that the direct methods result in a higher background, but this problem was not encountered in this study. However, when the fluorescence signal is weak, the CCD camera requires a longer exposure time

for image capture resulting in the capture of background surrounding the chromosomes.

4.1.5 Probe size

From the first description of CGH up until now, it has been emphasised that the size of the genomic DNA probes is a very important factor for CGH quality. It is a part of almost all CGH protocols to measure and adjust the size of the labelled DNA before hybridisation. It was found in this study that after optimisation of the CGH technique in the lab, the size of the labelled DNA is usually in the desired range. Therefore, measurement of probe size is not necessary in each experiment. This finding again reduces the time required for the experiment and makes CGH more practical for routine use.

4.1.6 Post-hybridisation steps

Post-hybridisation steps applied in the modified protocol described in this study are sufficient and less complex than published protocols which again reduces the time required for each experiment. This protocol is similar to those used in current standard FISH protocols.

4.1.7 Image analysis

According to this study, chromosomal imbalances can be detected by visual inspection of CGH images followed by global analysis of the fluorescence intensity ratios. When no abnormality is suggested further profile analysis was shown to reveal no previously undetected abnormality. As a very high resolution cooled CCD camera was used in this study for the capture of images, it is not clear whether lower resolution cameras can provide such images adequate for visual analysis. However, a statistical profile analysis is highly advantageous for detailed analysis of small abnormalities.

Until the very last stages of this study the IP-LAB software with SmartCapture extension was used for the image analysis.

Scripts written after each experiment were used to semiautomate the background removing, segmentation and normalisation steps. This measure was very time- saving. As it takes about 10-12 minutes for each image to be processed manually, a 20-image set required about 3-4 hours to process. However, using the new Quips software (or equivalent software) and computers with faster processors each image is processed in a few minutes, and thus there is no need for this facility in standard CGH analysis. As is discussed later, SmartCapture software may still be preferred for the analysis of telomeric regions.

4.1.8 The modified protocol

In summary, the modified protocol provided more reproducible, less complex CGH experiments. Meanwhile, images obtained using the modified approach were of higher quality and had lower background. This protocol follows the 48-72 hours hybridisation time but uses shorter post-hybridisation steps. As the post-hybridisation steps are less complex and very similar to those are already applied for FISH, it is familiar in the in situ lab.

4.2 Test of modified protocol using known cytogenetic abnormalities

There was complete agreement between the CGH and conventional cytogenetics on all samples of the series of known cytogenetic abnormalities examined in this part of the study. Thus, it is evident that CGH can provide an alternative method of detecting common chromosome abnormalities when there is difficulty in obtaining good quality metaphases.

CGH may be helpful in analysis of samples from aborted foetuses or tissues from still-born babies with suspected chromosomal abnormalities. CGH may even be helpful in detecting unbalanced chromosomal aberrations present in frozen stored samples from many years ago. Many samples used in early stages of this study were obtained from cells frozen since early 1970s. These

samples could not be reconstituted, but the DNA contents of the cells proved applicable for study.

Another application of CGH would be the detection of common numerical abnormalities in a prenatal diagnosis service. This approach is comparable to interphase cytogenetics and much faster than the conventional AF culture which takes more than 2 weeks. It would be useful only for unbalanced abnormalities as CGH is unable to detect balanced aberrations. While this is an important disadvantage, it does not have a practical impact as pregnancies carrying balanced abnormalities are allowed to continue, especially when parents with the same aberrations have no clinical symptoms. Moreover, in pregnancies suspected to carry balanced aberrations, when appropriate tissue is available, interphase FISH would be the technique of choice.

Although the resolution of the standard CGH is almost the same as that of the G-banding, CGH could be useful in circumstances in which the banding pattern has not changed but an unbalance aberration is present.

4.3 Improving limits

4.3.1 Mosaic supernumerary ring Chromosome 19 identified by CGH

So far the approach to determining the origin of cytogenetically unidentifiable chromosomes has involved stepwise FISH using forward painting methods with alpha-satellite, single-copy or whole chromosome painting probes, or reverse chromosome painting procedures with either flow-sorting or microdissection of the particular chromosome (Blennow et al 1992; Carter et al 1992; Magnani et al 1993; Lengauer et al 1991; Deng et al 1992; Ohta et al 1993; MullerNavia et al 1995; De et al 1996). Microdissection followed by DOP-PCR amplification of the dissected DNA is increasingly being used and its modified approaches are reported to be so efficient that just one dissected DNA fragment is sufficient for a successful analysis (MullerNavia et al 1995). However, not all diagnostic

cytogenetics laboratories have easy access to microdissection technology. In the case reported an alternative approach which is not dependent on patient metaphase and is a genome-wide screening technique enabling the result to be achieved in just one experiment. Many image analysis systems now in use in cytogenetic laboratories include software for CGH, and results of analysis are available in three working days, which is comparable to the time required for microdissection. Where further confirmation by FISH is necessary one more day is required.

An advantage of CGH over microdissection is that as demonstrated here, it can provide an estimate of the proportion of cells containing the ring chromosome in the tissue studied. This information is laborious to obtain by chromosome analysis or interphase FISH. The minimum percentage of abnormal cells that can be detected by CGH has not yet been defined but based on this report, as low as 28 percent mosaicism in cultured metaphase cells could be detected. Theoretically, employing a medium restrictive fluorescence ratio of 0 .75-1.25, there should be at least 50% abnormal cells present for the chromosome imbalance to be detected (du Manoir et al 1995 a, b; Piper et al 1995). In the present case, fluorescence ratio 0.8-1.20 was the most restrictive threshold at which the abnormality was detected, which leads to an estimation of the percentage of abnormal cells in uncultured blood of at least 40%. This proportion differs from the result of cytogenetic analysis in cultured cells which is based on a small sample of metaphases obtained from PHA stimulated T-lymphocytes, and which showed not more than 28% abnormal cells.

Since in CGH the centromeric regions are suppressed, the method would not be applicable to ring or marker chromosomes composed almost entirely of centromeric heterochromatin.

In the case described here no short arm material could be demonstrated in the ring chromosome either by CGH or by FISH using short arm paint, suggesting a breakpoint at or immediately adjacent to the centromere. No more detailed studies have yet been undertaken to define this breakpoint more precisely. CGH

studies indicate that virtually the entire long arm of chromosome 19 is present in the ring, although by FISH the telomeric region is shown not to be present. As is shown in this study, partial trisomy or partial monosomy of as little as 4-5 Mb can be detected in telomeric regions by CGH. The fact that CGH has not shown any variation in copy number of any part of 19q suggests that the break-point is within 5 Mb of the telomere, and that the loss of long arm material in the formation of the ring chromosome is beyond the resolution of CGH.

The first series of FISH experiments using empirically selected centromeric probes including one for chromosome 19 failed to reveal the origin of the ring. This may be explained in part by the low percentage of cells containing the ring, and difficulties in identifying it with certainty in some mitoses. Also the alpha satellite probe hybridising to chromosomes 1, 5 and 19 was used in the study since at that time it was the only available centromeric probe for chromosome 19. It is possible that a small change in stringency conditions caused some loss of signal for chromosome 19.

As discussed above, when no cytogenetic or clinical clues are available, a multicolour or stepwise FISH approach with or without chromosome microdissection may be employed to identify the origin of a marker chromosome. However, in investigating small marker chromosomes, many service laboratories presently carry out sequential experiments employing FISH probes that are selected empirically. This experience indicates that in FISH studies the empirical approach may not detect a marker that is present in a lower percentage of metaphases even although it is detected subsequently in a targeted FISH study. Although this experience is unlikely to be a frequent occurrence, it is one that is important for clinical cytogenetic practice.

4.3.2 CGH for cryptic translocation screening in patients with idiopathic mental retardation

A cryptic translocation is one which cannot be observed by conventional cytogenetic analysis, either because the size and banding pattern of the

chromosome regions involved are too similar for the exchange to be detected, or because the size of the exchanged segments is close to the limit of resolution of the cytogenetic technique (Lamb et al 1989; Overhauser et al 1989; Ledbetter 1992; Wilkie et al 1993). Small translocations involving telomeric regions of chromosomes are especially difficult to detect since most telomeres have a similar banding pattern. To date, detection of cryptic telomeric translocations has mostly relied upon the presence of clinical or cytogenetic clues that point to a specific chromosome deletion or duplication syndrome. The availability of DNA probes that permit informative DNA or molecular cytogenetic studies is also crucial. To diagnose cryptic translocations without a specific clue to the chromosome segment involved requires a genome-wide screening technique.

The data presented here show that modified CGH analysis can detect unbalanced cryptic, telomeric translocations of at least some chromosomes. Previously, data relating to the telomeric regions of the chromosomes have always been excluded from CGH analysis (Kallioniemi et al 1994), mainly because the green and red fluorescence intensities gradually decrease at the chromosome telomeres, and therefore, a reliable colour ratio is not possible with the currently used algorithms. However, by introducing a new strategy involving close attention to previously ignored telomeric regions and the application of a highly restrictive threshold, we have been able to demonstrate translocations involving the telomere of 2q and 17q, 20p and 4q, 20q and 10q.

It was noted that partial trisomy was more readily demonstrated by the modified technique than was partial monosomy. This phenomenon may be due to the behaviour of the fluorescence intensities at the chromosome terminals: when an extra copy of DNA from a telomere is present, this may compensate for the decreasing fluorescence intensity at the chromosome end and the signal shows a sharp rise, while in contrast, the intensity of signal corresponding to control DNA gradually decreases at chromosome ends. When this pattern is present and the above criteria are adhered to, diagnosis of DNA gain corresponding to at least one extra telomere is possible. Conversely, in partial monosomies with loss of one copy of the terminal band, there is less difference in the patterns of

fluorescence intensity in control and test DNA. While the intensity of the test signal decreases sharply, the fluorescence intensity of the control signal increases slightly at first and then gradually decreases. The initial slight increase might be explained by increased hybridisation of control DNA when it competes with less test DNA from the deleted chromosome segment. This difference in the gradient of the fluorescence intensities, although less obvious than that associated with the partial trisomies, was sufficient to lead the detection of DNA deletion in the three families described here.

There is no reported evidence that the detection of partial monosomies using CGH has proved difficult, at least with respect to larger sizes of DNA loss. On the other hand, DNA losses of the size seen here, especially those involving telomeric areas, have only rarely been described using other techniques and a study of the sensitivity of CGH in this situation is warranted. Results to date appear to justify using the modified CGH technique to screen a larger series of patients where clinical genetic evidence points to cryptic rearrangement.

Thus when the standard CGH approach detects gain or loss of DNA material of one particular telomere, this observation should not be discounted in the following circumstances:

1. In cases of gain of DNA material:

a) using a restrictive threshold, global CGH analysis should show similar findings in both homologues in most metaphases;

b) intensity of fluorescence corresponding to test DNA at a telomeric area should increase, while fluorescence intensity of control DNA decreases.

2. When loss of DNA material is suggested, the intensity of fluorescence corresponding to the control DNA increases slightly at first and then decreases, but fluorescence intensity of test DNA decreases sharply from the beginning of the deleted segment. These patterns must be present in the averaged profile as well as in most individual chromosome profiles. Targeted FISH studies using appropriate probes are then employed to confirm the CGH results.

Another approach to genome-wide analysis is provided by combinatorial multi-fluor FISH (M-FISH) (Speicher et al 1996) or spectral karyotyping (SKY) (Schrock et al 1996) where up to 27 different chromosome paint probes (including some arm-specific paints) have been used. Whole chromosome FISH probes can facilitate the detection of some small rearrangements, but telomeric regions are often under-represented in the painting probes (Rosenberg et al 1992).

During the time of this study telomeric probes for all human telomeres have been developed [National Institutes of Health and Institute of Molecular Medicine Collaboration, 1996) and may prove more informative using M-FISH or a multiprobe coverslip (Knight et al 1997) device. However, to cover the telomeric areas of all chromosomes in M-FISH, 48 different telomeric probes in at least 24 different colours (ideally 48 colours) are required (Ledbetter 1992) and the multiprobe coverslip device requires a high quality chromosome preparation of high mitotic index. Although the device has demonstrated the presence of known abnormalities, it has not yet been tested in a blind study. With both methods, subtle cryptic deletions outside the telomeric area may remain undetected.

The fact that the highest gene concentrations in the human genome are in telomeric bands of metaphase chromosomes (Saccone et al 1992) makes the study of these abnormalities particularly important for understanding the causes of mental handicap, a common problem which affects up to 3% of all school-age children and is of idiopathic or unexplained origin in up to 80% of cases (Roeleveld et al 1997; Yeargin-Allosop et al 1997). Recently, Flint et al found 3 cases of cryptic *de novo* rearrangements among 99 patients with idiopathic mental handicap who were screened for subtelomeric chromosomal abnormalities with a combination of molecular cytogenetic and DNA techniques (Flint et al 1995). All three cases diagnosed in that report had *de novo* rearrangements whereas the patients described here were selected for detailed study because inheritance patterns and clinical features strongly suggested they suffered *familial* chromosomal imbalance. The patients presented in this study

also had larger, cryptic rearrangements (in the range 4-10 Mbs) which were only detected after CGH was adapted to work successfully at its highest reported resolution in clinical cytogenetics, close to its ultimate theoretical resolution using the current available technology.

Although CGH analysis is presently unable to detect abnormalities less than 4Mb in size, given the discovery of the important contribution of larger cryptic rearrangements to familial, idiopathic mental handicap, there is a high priority to investigate the use of CGH as a tool for genome-wide screening in a larger series of sporadically affected individuals with idiopathic mental handicap.

4.3.3 CGH for hyperploid tumours

Absolute intensity is based on various parameters from the DNA samples to target metaphase and optical setting as well as the real copy numbers. Thus, normalisation is carried out to compensate the effect of all other parameters except the copy number differences. The current approaches for normalisation are based on the assumption that because the same concentrations of the test and the control DNA are used, the fluorescence intensity corresponding to them should also be the same.

Table 4. 1 Main parameters in absolute fluorescence intensity

Illumination brightness
Filter bandwidths
Dye absorbance
Quantum efficiency
Camera sensitivity
Exposure time
Bleaching hybridisation efficiency
DNA concentration

The total green/red fluorescence intensity after normalisation is 1. Each chromosome will contribute to the total fluorescence intensity based on its chromosome contents. For example, in a normalised CGH image corresponding to a karyotypically normal test DNA, chromosome 1 constitutes about 8% of the total fluorescence value after normalisation. This figure is deduced from relative length of this chromosome with respect to total diploid genome.

In case of trisomy 1, DNA sequences corresponding to this chromosome constitute about 12% of the total diploid DNA and therefore 12% of the total fluorescence value. This increase occurs at the expense of a reduction in the percentage of all the other chromosomes. In near triploidy, trisomic chromosomes constitute a higher percentage of the total DNA at the expense of a larger decrease in the percentage of normal chromosomes. This phenomenon results in a misleading ratio profile showing a loss of DNA corresponding to the normal chromosomes. However, if the CGH analysis is performed on the fluorescence ratio corresponding to chromosomes with known copy numbers this misinterpretation will not occur.

4.3.4 Overnight CGH

Standard CGH protocols are based on protocols which require 2-4 days of hybridisation. Routine CGH applications are not critically dependent on time. Therefore, a shorter CGH protocol may not have a very important impact on routine CGH applications. On the other hand the protocol presented in this study is mainly based on increasing of the amount of DNAs and fluorochromes used and therefore is more expensive.

In newborns with severe malformations in life threatening conditions or newborns with ambiguous genitalia a chromosome analysis is required for

proper diagnostic and therapeutic procedures. Chromosome analysis based on direct preparations from bone marrow samples or more recently from uncultured neonatal blood are used for rapid cytogenetic analysis. In certain conditions however, metaphase chromosomes are not available and therefore overnight CGH may be helpful.

4.4 Reinterpretation of a putative isochromosome Xp

Cytogenetic analyses show that the region Xq11.2-q21 is retained in all structurally abnormal X chromosomes. True isochromosomes for Xp probably do not exist in a liveborn. There are no non-mosaic reported cases with complete duplication of i(Xp) confirmed by FISH or molecular techniques. From such observations the conclusion is drawn that this 'critical region' contains the locus controlling X inactivation. Structurally abnormal X chromosomes without the X-inactivation centre would allow nullisomy, disomy, or trisomy for genes on the X chromosome--presumably nonviable states.

Brown et al (1991) describe *XIST* (X-inactivation specific transcript) gene which is expressed specifically from inactive, but not active, X chromosomes. By in situ hybridisation, the *XIST* gene is located in band Xq13, at the interface of bands Xq13 and q21.1.

The true chromosomal constitution of the case with putative isochromosome Xp presented in this study, was detected in a similar manner to that described for the case of ALL with abnormal chromosome 9 (4.6.1).

Visual CGH showed that the intensity of fluorescence signals corresponding to Xq centromere to Xq21 of the test DNA was the same as the signal corresponding to that segment of the control DNA. Moreover, profile CGH analysis showed a 1/1 test/control ratio at this segment when a normal female had been used as control and 2:1 ratio when a normal male had been used as control, confirming that the patient has more copies of this segment than a normal male has.

This confirmed the presence of Xq segment and *XIST* region in the abnormal X chromosome. This experience shows again the ability of CGH as a complementary technique to the current techniques.

4.5 A case of follicular lymphoma with double minutes and complex cytogenetic abnormalities

A case of follicular lymphoma is presented in which multiple abnormalities in the absence of t(14;18) were present. Chromosome 1q and 12q duplications are reported to be correlated with low grade and low-intermediate clinical aggressiveness (Yunis et al 1987). On the other hand double minutes and abnormalities of chromosome 3 found in this case are reported to be accompanied with poor prognosis (Yunis et al 1987).

Double minute chromosomes are structures indicating gene amplifications which are frequently found in certain solid tumour types. Double minutes are a rare finding in follicular lymphoma. Among three large series analysed for cytogenetic abnormalities in follicular lymphomas, only Yunis et al (1987) found 4 patients with double minutes or homogeneous staining regions (HSR) out of 71 patients they studied. No cases of follicular lymphoma with DMs or HSRs were found among the patients studied by Tilly et al (1994) nor those studied by Avet-Loiseau et al (1997). On the other hand Yunis et al (1987) found a +3 or duplication 3p in 14% of the cases they studied, mostly among those negative for t(4;18) [7/11 in t(14;18) negative cases compared to 3/60 in t(14;18) positive one].

Several abnormalities found in this case had remained undetected by G-banding and were identified only when CGH localised them on target metaphases. Since both chromosome 8s were normal in G-banding and no landmarks were present on the additional material on 3p, this abnormality was detected only when CGH found a gain of DNA material corresponding to 8q. Targeted FISH then confirmed that the additional material originated from chromosome 8.

Abnormalities found on chromosomes 1 and 12 also were resolved with the help of CGH and confirmed by targeted FISH.
As double minute chromosomes play a critical role in tumour cell genetics where they are frequently associated with the over expression of oncogene products (Hahn, 1993), the 3p26 amplification found in this case may present a candidate locus for a possible oncogene at this site.

4.6 CGH for the investigation of haematological malignancies

In the present study, 14 patients with haematological malignancies were examined, and the results were compared with karyotypes obtained by banding analysis. In 2 cases CGH did not detect the abnormality found by conventional cytogenetics because of the balanced nature of the rearrangements. Both CGH and conventional cytogenetics found no abnormality in 5 samples. In 3 abnormal cases CGH and G-banding results were compatible. In 3 patients CGH detected abnormalities missed by banding technique.

4.6.1 A case of ALL with 9p;9q translocation

In the first case, after detection by CGH of partial gain and losses for 9p and 9q, retrospective analysis of the metaphase chromosomes from the patient confirmed the existence of an abnormal chromosome 9 in 2 metaphases. Standard banding suggested that the abnormal chromosome 9 was an isochromosome and a referral lab confirmed that diagnosis. However, this diagnosis was based on only two very low quality metaphases and was not compatible with the CGH finding which showed a small segment of chromosome 9p to be present. This discordance could not be resolved as no metaphase chromosome was available from the first sample and the next sample revealed no abnormality because the patient had already undergone therapy. This discordance was resolved one year later when the patient had a relapse. Chromosome analysis from a metastatic tumour showed an abnormal

chromosome 9, with two equal size arms, very similar to an isochromosome 9. But the G-banding results suggested a 9p;9q translocation leading to a partial monosomy/trisomy of 9p and 9q respectively. The derivative chromosome as suggested by G banding was: der (9)(qter->q22-34::p22-> qter). As shown, the banding pattern of the suggested derivative chromosome is very similar to that detected by the CGH. Although the similarity of the banding pattern is a real problem in G banding analysis, this is not an issue in CGH.

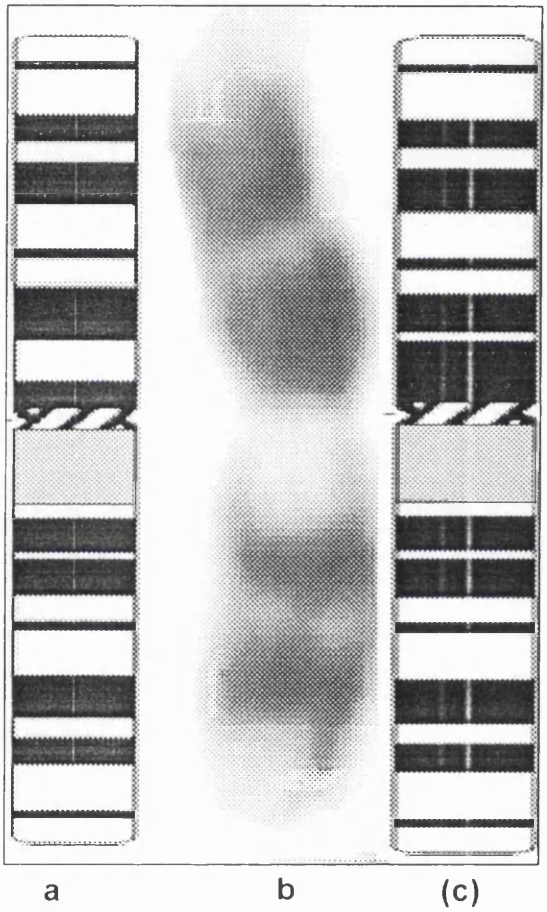


Figure 4. 1: (a) The derivative chromosome suggested by G banding (b) Digitally magnified image of the abnormal chromosome 9 discussed here. (c) The derivative chromosomes suggested by CGH. As shown, the banding pattern of the chromosome is similar to (c). This finding together with FISH results confirms the CGH findings.

The breakpoint suggested by the G-banding was more distal than that suggested by CGH. FISH however confirmed the CGH findings. As G banding is dependent on the light and dark banding pattern present on the chromosome arms, this technique is unable to detect the abnormality when the banding pattern is not changed, or the quality of the metaphase spread is not high enough to define the precise breakpoint.

The CGH finding of the deletion of 9p at 9p12 is compatible with the fact that deletion of 9p 21-22 is associated with about 7-13% of ALL. More studies to determine the precise molecular nature of the abnormality was not performed as that was not an aim of the study.

4.6.2 A case of AML with loss of the Y chromosome

Standard CGH showed a loss of Y chromosome, which was compatible with that of the G banding. Visual analysis of the CGH image, however, showed green signals corresponding to the test DNA on the target Y chromosome. In case of complete loss of the Y chromosome there should have been no signal corresponding to the Y chromosome. The presence of the signal corresponding to Y chromosome confirms the existence of cells containing Y chromosome sequences. There are at least two theoretical explanations for this matter. First, although no Y chromosomes are present in the cell compartment there are some Y chromosomes sequences inserted into other chromosomes. Another explanation would be a presence of a population of cells with normal karyotype missed by chromosome analysis. The second explanation looks more likely. As no additional material was available, FISH or other confirmatory technique could not be performed.

The loss of the Y chromosome is a feature found in haematological malignancies but it is also found in normal bone marrow in elderly males. The degree of loss of Y (% -Y cells per patient) increases with age in normal individuals but not in patients with MDS or AML. Therefore, in elderly men -Y is not indicative of malignancy and should not be considered as a marker of the

malignant clone. This finding therefore has mostly a technical impact rather than a diagnostic one.

In approximately 5-10% of haematological malignancies, cytogenetic analysis is not successful because of poor preparation quality or low mitotic index (Success rate figures from U.K. NEQAS 1995/6). On the other hand normal karyotypes are found in 40% of cases in large prospective studies investigating acute myeloid leukaemias, which, at least in part, is due to mitotic cells arising from nonlukaemic cells (Bloomfield and de la Chapelle, 1987).

This study shows that CGH can play an important role as an additional tool in cytogenetic analysis. As CGH is not dependent on dividing cells it has, with certain limitations, the potential to distinguish true chromosome gain or loss from complex karyotypes. CGH has also the ability to pinpoint the origin of chromosome gain or loss in cases with simple unbalanced abnormalities. This advantage simplifies the selection of chromosome probes for further FISH studies. In rare cases, double minutes are present in myeloid leukaemias. By CGH, the chromosomal localisation of such amplified sequences can readily be determined and this can lead to the identification of a specific gene being amplified. CGH can also provide a way forward in cases where cytogenetic analysis is unsuccessful or not possible. As a well established technique for genome wide screening, CGH may help to uncover masked or underlying abnormalities, and in this respect it is important that successful results can be obtained from volumes of samples as low as 0.3 ml.

4.7 CGH using universally amplified DNA samples

The combination of CGH and DOP-PCR in this study confirms the earlier reports regarding the use of universally amplified DNA for CGH. DNA samples as low as 1 ng were used in this study.

Speicher et al (1993) reported a technique based on CGH and DOP-PCR which allowed the detection and chromosomal localisation of DNA sequence copy

number changes in solid tumor genomes from frozen sections and paraffin embedded, formalin fixed specimens.

Wiltshire et al (1995) used DNA extracted from microdissected tumour for DOP-PCR. The present study shows the feasibility of using this approach in conditions where sufficient samples are not available for a cytogenetic analysis. The quality of images were not comparable to those performed by standard approach but sufficient for detection of copy number differences.

Contamination of the test DNA with non-target DNA is theoretically a major issue in DOP-PCR. In the present study contamination was not a serious problem as all necessary measures had been taken to prevent such problem. Moreover, the amount of DNA used in this study was more than used in those other reports since a washed out sample from a tube yielded more than 1 ng DNA.

There are some recent reports of using of universally amplified DNA from single cells for preimplantation diagnosis. In these situations contamination is potentially a serious problem.

4.8 DNA molecules as hybridisation targets

As metaphase chromosomes are used as the hybridisation targets CGH is limited to a resolution similar to that of the G banding technique. In theory, the resolution of CGH could be improved by targeting the extended chromatin targets similar to the high resolution G-banding. This approach has not yet been successful because the degree of overlapping of metaphase chromosomes causes great difficulties in CGH analysis. Targeting of single or high-density DNA fibre molecules, or high density DNA microarrays was thus carried out to test the feasibility of high resolution CGH.

4.8.1 Comparative hybridisation to DNA fibre targets

The feasibility study carried out with the cosmid clone shows a direct correlation between the ratio of concentrations of differentially labelled probes and the ratio of their corresponding fluorescence intensities. An immediate conclusion would

be that this supports the concept that the copy number differences of genes between test and control DNAs should be detectable. The results of this study are in accordance with those recently reported by Kraus et al (1997).

In standard FISH as low as 1 ng of labelled probe is used to detect its corresponding sequence in the target cell. This volume of probe containing an extremely high number of DNA copies makes it very likely that the hybridising probe will find its counterpart in the target cell. In standard CGH however, it is not possible to have so many copies of each diploid genome.

The cosmid probe used here is about 35 kb in length. This size compared to the whole haploid genome is 1/100000 (35 /3000000). This means that 100000 times higher concentration of the genomic probe for each hybridising DNA should be used to keep the hybridisation as efficient as the comparative cosmid hybridisation. Based on the results of this study in which as low as 0.1 ng of cosmid probes in hybridisation mixture resulted in acceptable results, a concentration of about 20 μg (2 x 10) of each test and control DNA is required for a successful CGH analysis. This concentration makes the hybridisation solution very viscous preventing an efficient hybridisation. The need for competitive Cot-I DNA makes the situation even less desirable.

There are more reasons which make the CGH to DNA fibres even less attractive. The main advantage of CGH is its ability to perform a genome-wide screening approach. A potentially successful CGH to DNA fibres lacks this advantage. Pre-knowledge of the segment to be analysed is required to prepare corresponding cosmid target for it. A statistical analysis of data corresponding to at least 10-20 fibres analysed is required to successfully detect the copy number differences in only one target. An immediate advantage for CGH to DNA fibres would be high-resolution assay of copy number of a particular segment of the genome. As a previous knowledge of the area to be screened is required other currently available techniques, like FISH using site specific probes, molecular techniques like RFLP or even certain blotting techniques would do the same analysis with less complexity.

The technical problems regarding the high concentration of the genomic probe required for the hybridisation may be overcome by different approaches. By substituting a single target with a pool of chromosome segments cloned in YAC, BAC, PAC, cosmid or other this problem may be overcome but at the expense of resolution. This approach requires preparing several DNA targets isolated from clones, and reduces the resolution down to the standard CGH. Another approach may be targeting high density DNA arrays from a single cosmid probe as used in this study.

4.8.2 CGH to dotted high-density DNA fibres

This study shows that different copy numbers of X chromosome can be detected by this approach and deletion or gains of DNA segments of about 35-40 kb should be detected. Moreover, by dotting more targets on the glass slide it is possible to screen many targets simultaneously. This is an advantage to the previous approach applied on DNA fibres which only one target could be analysed. Furthermore, fewer spots are required compared to 10-20 fibres which are required by the previous approach.

While this study was underway, Solinas et al (1997) reported obtaining successful results using a similar approach. They used chromosome-specific libraries or human DNA fragments cloned in PAC vectors, 75-130-kb in length as target DNA and detected the hybridisation signals by confocal laser scanning microscope. The highest resolution they reported was about 75 kb which is much lower than that achieved in this study.

4.8.3 CGH to DNA microarrays

Targeting PCR products for hybridisation can be described as a modified reversed dot blot technique. In the reversed dot blot assay, PCR amplified test DNA is hybridised to the probe dotted on a hybridisation membrane. Main differences between the reverse dot blot and the hybridisation technique carried out in this study is that the genomic DNA instead of PCR amplified DNA is used

in the method described here. Another difference is that a glass slide instead of a cellulose or plastic membrane is used to immobilise the target DNA. Moreover, probes are labelled by fluorochromes and a differentially labelled control genomic DNA is co-hybridised with the sample to be tested. However, this method is not designed to detect point mutation.

The results described in this study show the feasibility of CGH to DNA array targets. This approach would improve the resolution of the technique up to 300 bp.

One important technical improvement was the use of high density DNA targets instead of low concentration DNA fragments. Another main factor is believed to be the hybridisation to single instead of double stranded DNA. This measure makes denaturation of the target DNA unnecessary and therefore prevents the DNA being washed out during the denaturation by 100 °C water or 70 °C formamide. Using DNA made by DNA synthesiser may provide a reasonable alternative.

Using the currently fast developing robot technology controlled by appropriate software makes it theoretically possible to target selected DNA fragments from the whole genome simultaneously. An ultimate progress would be a barcode of 400-800 DNA fragments from all over the genome spotted in a known order on a glass slide or an appropriate membrane. Such barcode may be screened in a single experiment.

4.9 Conclusion and future prospects

CGH is a powerful adjunct to conventional banding methods and FISH in many fields of clinical cytogenetics. These fields include constitutional chromosome aberrations and cancer cytogenetics. CGH may detect some abnormalities overlooked by current techniques. CGH can be used to help current techniques to reinterpret or define the origin of marker chromosomes and isochromosomes and detect cryptic chromosomal aberrations. CGH may also help to define the

origin of double minutes chromosomes in samples from malignant tumours and leukaemias.

It is not unrealistic to say that in the near future it will become routine to scan automatically by CGH to DNA biochips the entire genome of individual patients in a single experiment.

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