

## **Technicolor Genome Analysis**

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- 1 INTRODUCTION
- 2 PRINCIPLES BEHIND FISH
  - 2.1 Preparation of Cytological Specimen
  - 2.2 Preparation of DNA Probes
  - 2.3 Labeling of DNA Probes
  - 2.4 Hybridization of Probe to Specimen
  - 2.5 Detection and Visualization
  - 2.6 High Sensitivity Detection Procedures
- 3 APPLICATIONS OF FISH
  - 3.1 Gene Mapping
  - 3.2 Somatic Hybrid Analysis
  - 3.3 Clinical and Cancer Cytogenetics
  - 3.4 Chromosome Evolution
- 4 KARYOTYPE ANALYSIS
  - 4.1 Comparative Genome Hybridization
  - 4.2 Spectral Karyotyping

## **10.1. Introduction**

Our understanding of the organization of complex genomes has progressed rapidly in the last fifty years. This has been the result of intense and dedicated work not only in the biological sciences but also chemistry and physics. Our current knowledge about genomes owes a debt of gratitude to the interplay between these three disciplines. The desire for a deeper understanding of the complex mechanisms of life has posed numerous technical challenges. Among these was the need for increased optical resolution. One of the best examples of the merging of the three sciences is the development of fluorescence in situ hybridization and its application to other newly developed techniques for the analysis of genomes. In this chapter we will discuss the combined application of chemically modified nucleosides and advances in microscopy, optics, digital imaging devices and image analysis software.

### **10.1.1. Historical Perspective**

The first published use of a nucleic acid probe for the in situ detection of its complementary sequence came from two different groups. Both Gall and Pardue (1969) and John, Birnstiel and Jones (1969) hybridized tritium-labeled *Xenopus* rRNA to cell squashes of ovarian tissue fixed on microscope slides. The specificity of the hybridization reaction was demonstrated through the use of such controls as treatment of the slide with DNase, not denaturing the cellular DNA prior to hybridization, treatment with RNase A to demonstrate the stability of the DNA/RNA complex, and competing the hybridization reaction with unlabeled rRNA. Despite the undesirable use of radioactivity and the long exposure times of 5 – 22 days, this was the first technique that could be used to “...assign the chromosomal location of a given DNA segment by means other than conventional cytogenetic and genetic analysis. Thus chromosomal mapping of sequences detectable by molecular hybridization is limited to a small number of genetically favorable organisms, or to rare viable deletion mutants, or to comparisons between the sex chromosomes of some organisms” (John et al., 1969).

Around the same time that in situ hybridization was being developed, chemists and biochemists had begun experimenting with different nucleoside analogs in the search for agents with antiviral and anticancer activity. One approach was to identify agents that would interfere with the biosynthesis or function of the viral nucleic acids without causing host toxicity. This could involve inhibition of viral genome replication or transcription, viral mRNA translation, viral enzyme catalysis or virus maturation. Perhaps differences in the bioavailability of the nucleoside analogs and differences in incorporation rates would result in sensitivity to these chemically modified DNA precursors. It was from these same chemists that attempts were made to improve in situ hybridization.

One of the first reported attempts to improve in situ protocols took advantage of the strong affinity between biotin and avidin (Manning et al., 1975). The removal of radioisotopes from the reaction increased the spatial resolution of the signal and indefinitely extended the shelf-life of the probe. Manning et al. covalently coupled biotin (vitamin H) to the rRNA probe via a cytochrome-c bridge. The efficiency of this reaction resulted in about one biotin-cyt-c label for every 100 – 200 ribonucleotides. This ratio, and the use of the small molecule biotin, remain important factors in trying to keep steric hindrance of the probe to a minimum thereby maintaining the hybridization kinetics. The synthesis reaction first linked the cytochrome-c and biotin molecules. This moiety was then coupled to the rRNA by reaction with formaldehyde. It is important to note that this labeling reaction was not specific for any nucleotide in particular. The in situ hybridization protocol followed that of Pardue et al. (1969) with a few minor modifications. To reduce non-specific background, the slides were blocked with cytochrome-c prior to detection with avidin-labeled polymethacrylate spheres. The positively charged cytochrome-c effectively bound to the negatively charged sites on the microscope slide, thus preventing interaction with the positively charged avidin. The avidin-spheres, each of which on average contained a few avidin molecules, were then directly visualized by scanning electron microscopy, abrogating the need for long photographic emulsion exposure times as previously required. Variations on this protocol included the replacement of avidin-spheres with the electron-opaque protein ferritin covalently coupled to avidin and labeling of the RNA probe with biotin via a diamine bridge instead of the polyamine cytochrome-c (Broker et al., 1978).

Researchers at Yale University were also working on the development of nucleoside analogs with antiviral activity. They used their antiviral approach of synthesizing nucleosides that were efficient substrates for polymerase to improve upon the above in situ hybridization procedure. As such, they covalently coupled a number of different potential probe determinants directly to either the purine or pyrimidine rings of different nucleotides (Langer et al., 1981). Once synthesized, these labeled nucleotides were then used in a nick-translation protocol. Briefly, the nucleic acid probe of choice was incubated in the presence of DNase I, DNA polymerase and free nucleotides. The nucleotide cocktail contained all four nucleotides (dATP, dCTP, dGTP and dTTP) plus a lower molar concentration of Bio-dUTP. DNase I causes single-strand breaks, or nicks, in the DNA backbone which are then repaired by the polymerase. DNA polymerase I has both 5' → 3' and 3' → 5' exonuclease activity which allows it to remove bases from both sides of the nick site. It is during the replacement of these bases by the DNA polymerase activity of the enzyme that the biotin-labeled nucleotide, as well as the others in the reaction, are incorporated. At the ratios of

DNase I to polymerase used, not all of the nicks are repaired, thus the size of the DNA probe is decreased, resulting in a smear on an agarose gel.

The size of the probe after labeling is important for both the hybridization kinetics as well as accessibility to the genomic target DNA. There is also a mixture of dTTP and dUTP in the reaction. Since dUTP is not a normal substrate for DNA polymerase, the incorporation efficiency of dUTP is less than for the other nucleotides and therefore not every thymidine residue is replaced with a labeled nucleotide. This is important for maintaining the proper hybridization kinetics as well as an efficient nick translation reaction.

These hapten labeled nucleotide probes were then used for detecting and localizing specific sequences in cell, tissue and chromosome preparations as well as membrane bound nucleic acids (Langer-Safer et al., 1982; Manuelidis et al., 1982). Biotin-labeled probes were initially detected using rabbit anti-biotin antibodies. These were either directly conjugated to a fluorescent molecule like fluorescein isothiocyanate (FITC) or were themselves detected with a peroxidase-conjugated sheep anti-rabbit IgG (Langer-Safer et al., 1982). There are currently many different directly-conjugated nucleotides available. Some are conjugated to fluorochromes like FITC, Spectrum Green, TRITC, Rhodamine, Spectrum Orange, Texas Red, Cy5, etc. while others are conjugated to non-fluorescent molecules like biotin, dinitrophenol (DNP) or digoxigenin that are detected with fluorescence-conjugated avidin or antibodies directed against them.

Fluorescence In Situ Hybridization (FISH) has revolutionized the way in which geneticists are able to analyze and visualize the genomes of various organisms. Research applications include the mapping of newly isolated DNA or RNA clones to their chromosomal locus and determination of the relative order of genes or clones in a contig with respect to each other. The fluorescence detection of nucleic acid probes has added to the repertoire of techniques already available for the analysis of genes and genomes, such as Southern blot hybridization and cytogenetic banding. Microscopic analysis of chromosomes hybridized directly with fluorescent DNA probes allows rapid mapping of genes and clones to their genomic chromosomal locus as well as sometimes identifying other loci containing related sequences. The positional relationship between different clones along the chromosome can also be readily determined. By labeling each clone with a different fluorescent molecule, their simultaneous hybridization to either condensed chromosomes or elongated chromatin fibers allows determination of order. The advent of different fluorescent dyes, suitable optical filters and imaging technology has increased the amount of information that can be obtained from a single hybridization. The culmination of these advances can be found in the technique known as Spectral Karyotyping (SKY), in

which 24 different chromosomes can be distinguished by their spectral signature after in situ hybridization.

## **10.2. Principles behind FISH**

In the general sense of the term, FISH involves the hybridization of fluorescently labeled nucleic acid probes to genomic DNA preparations on a microscope slide. Since there are many different types of specimens and probes, other names have been given to the more specific applications of this technique. FISH is used primarily to refer to the use of probes shorter than a few megabases and chromosome painting probes to metaphase chromosome preparations. The typical application of FISH is for the genomic localization of particular probes (i.e. gene-specific plasmids, cDNA, ESTs, YACs, BACs, PACs) by physical mapping onto metaphase chromosomes (Fig. 1A, B & E). Interphase FISH, sometimes referred to as interphase cytogenetics, involves the visualization of chromosome aberrations in cell nuclei by hybridization with nucleic acid probes. This technique was first described in 1986 when Cremer et al. used radiolabeled probes to detect the copy number of chromosome 18 in normal nuclei and nuclei with a trisomy 18. Specimens range anywhere from fresh cytological preparations to archived paraffin-embedded, formalin-fixed tissue sections. The replacement of radioactivity with fluorescence made the technique more amenable to use in a clinical setting and also increased the resolution of the assay. Interphase mapping is used to determine the relative order of specific probes with respect to each other (Fig. 1C). This is typically employed when the distance between a number of probes is below the limit of detection using metaphase chromosomes. Another technique with the same application, but higher resolution, is fiber FISH (Fig. 1D). In this strategy, the nuclear membrane is perforated using detergents and the uncondensed genomic DNA “spills” out of the nucleus and spreads along the surface of the slide as long DNA filaments. Since the DNA is arranged two-dimensionally, as opposed to its three-dimensional arrangement in the nucleus, the resolving power in terms of physical distance between two loci is increased (Monier et al., 1998; Ried et al., 1995; Schwartz and Samad, 1997; Tocharoentanaphol et al., 1994; Wiegant et al., 1992) Thus, due to the diverse applications of FISH, there are many variables involved in the preparation of specimen and probe as well as the exact protocol used for hybridization and detection of the annealed probe.

Figure 10.1

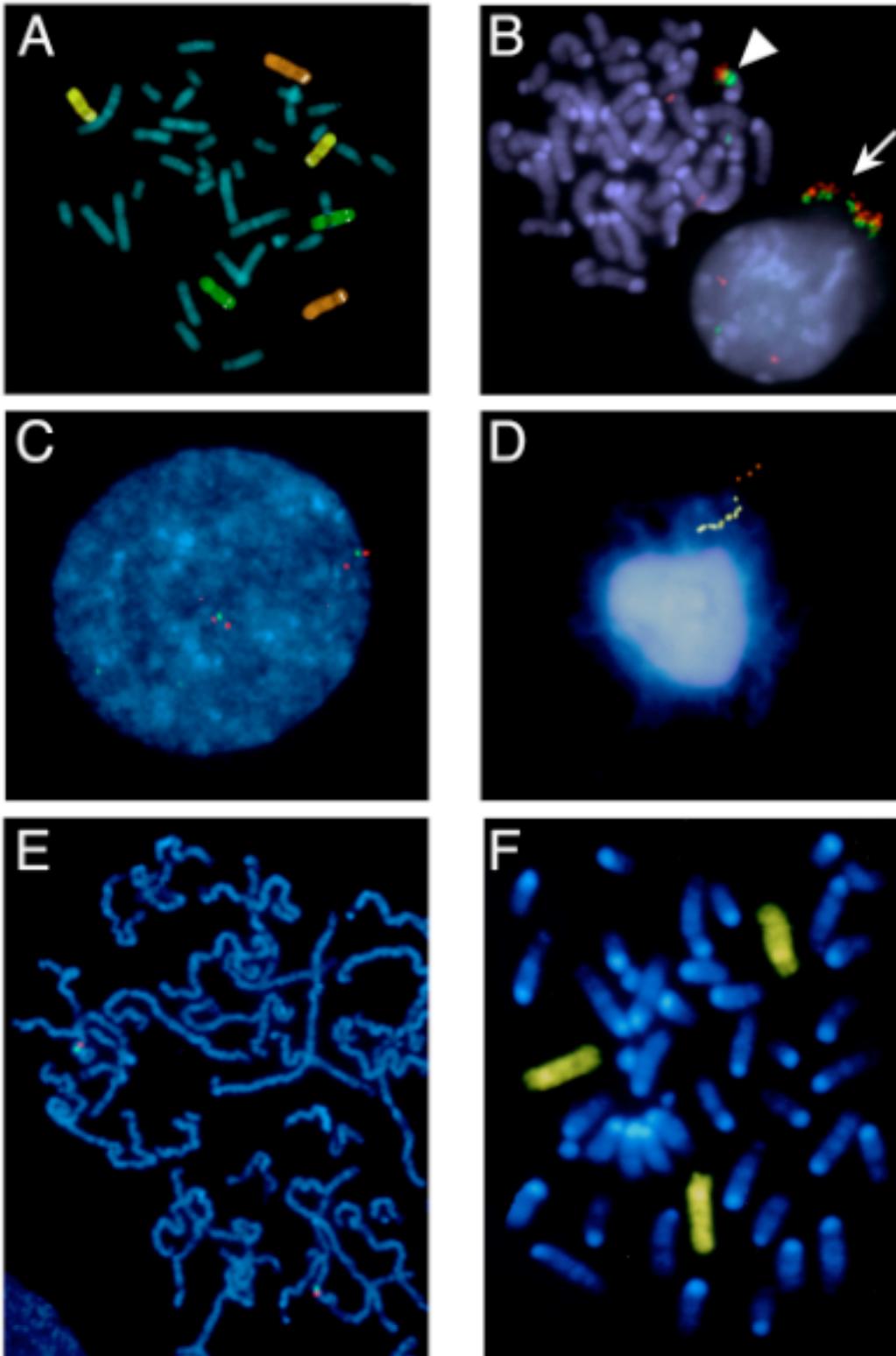


Figure 10.1:

- A. Fluorescence in situ hybridization of painting probes specific for human chromosomes 5 (green), 7 (purple) and 12 (yellow) and gene-specific probes for the mammalian bitter taste receptors (M. Difilippantonio & N.J.P. Ryba).
  - B. Mouse tumor metaphase chromosomes hybridized with probes specific for the immunoglobulin heavy chain locus (red) and the c-myc gene (green). A translocation between the chromosomes on which these genes reside has also resulted in amplification of the genes on the derivative chromosome (arrowhead). This is also evident in the adjacent interphase nucleus where this DNA leaked out of the nuclear membrane during the processing of the slide (arrow). The other loci appear as discrete dots in the nucleus (M. Difilippantonio & A. Nussenzweig).
  - C. Interphase mapping of three gene-specific probes reveals that the chromosomal position of the gene detected with the FITC-labeled probe (green) is between the two genes detected with the TRITC-labeled probes (red) (courtesy of R. Yonescu).
  - D. Biotin (green) and digoxigenin (red) labeled probes specific for two halves of exon 45 in the Duchenne's muscular dystrophy gene can be spatially resolved on chromatin fibers using DNA fiber FISH extracted nuclei (M. Difilippantonio & T. Ried).
  - E. High resolution FISH mapping of two BAC clones corresponding to adjacent regions of DNA separated by approximately 1 Mb (courtesy of R. Yonescu).
  - F. Hybridization of a human chromosome 4 painting probe (yellow) to metaphase chromosomes from a human-hamster hybrid cell line reveals the presence of three copies of the human chromosome among the hamster genome (M. Difilippantonio & L.A. Doucette-Stamm).
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### 10.2.1. Preparation of Cytological Specimens

As indicated previously, there are many different scenarios in which one would like to detect specific DNA sequences. Most techniques involving in situ hybridization require the preparation of metaphase spreads as either the reference material (that to which the probes being analyzed are hybridized) or the actual sample itself. This is true for gene mapping, somatic hybrid analysis, determination of orthologous regions between species, spectral karyotyping (SKY) and comparative genome hybridization (CGH). In each case, the cells from which the chromosomes are to be prepared are treated with an agent that arrests the cells in mitosis, thereby increasing the number of cells containing condensed chromosomes. The most commonly used drug is colchicine, or Colcemid, which interferes with the progression through mitosis by inhibiting spindle fiber formation. There is a delicate balance that must be reached, however, between the number of mitosis and the length of the chromosomes, and these two parameters are inversely related. The longer one leaves the cells in Colcemid, the more cells will reach the mitotic block. The longer cells are

blocked, however, the more condensed the chromosomes become. Why then would one want to prolong the mitotic arrest? Some cultures grow particularly slow and therefore have very few cells entering mitosis at any given time. By increasing the Colcemid time (and usually decreasing the working concentration) one is able to increase the mitotic index of a particular harvest. If the number of mitosis is more important than the resolution of physical distance on the chromosomes, this may be a reasonable compromise. The length of treatment with Colcemid must therefore be experimentally determined for each cell type.

Other treatments are added depending upon the cell type and length of chromosomes desired. Human lymphocytes are usually stimulated to divide by addition of Phytohaemagglutinin (PHA) to the culture media. Mouse spleen cultures are stimulated with a mixture of lipopolysaccharide (LPS) and Concavalin A (ConA). Some protocols use methotrexate (MTX) to inhibit DNA synthesis by interfering with the synthesis of the deoxyribonucleotide thymidine triphosphate (dTTP). This has the effect of synchronizing the cells in S-phase. The cells are then released from the block for a predetermined period of time (usually 5 hrs) and allowed to cycle until they reach mitosis, where they are blocked with Colcemid and harvested. Another variation of this approach includes the addition of the nucleotide analogs BrdU and FudR after release from the S-phase block. Incorporation of these thymidine analogs into the newly synthesized DNA results in decreased condensation of the chromosomes and therefore tends to be used for high-resolution gene mapping.

Following mitotic arrest, the cells are resuspended in a 0.075M potassium chloride solution (KCl). Since this solution is hypotonic with regard to the concentration of KCl within the cells (i.e. the cells contain a higher concentration of KCl), water enters the cells in an attempt to restore the osmotic equilibrium. The net effect is that the cells swell causing the cytoplasmic membrane to burst, leaving only the nucleus intact. They are then fixed with a 3:1 mixture of methanol:acetic acid and dropped onto microscope slides. Although this seems like a very simple cookbook recipe, it is difficult to obtain high quality metaphase spreads. Parameters involved in obtaining a high quality slide include the length of time in hypotonic solution, the freshness of the fixative and the number of times it was changed, the cell concentration, and the humidity and slide drying time (a function of temperature). All of these variables affect the degree of spreading (so that as few chromosomes as possible are overlapping), the amount of cellular debris surrounding the chromosomes and the “hybridizability” of the chromosomes themselves. Removal of cytoplasmic and nuclear debris by treatment with Proteinase K and/or pepsin decreases the amount of background noise caused by nonspecific adherence of probe to the proteins, lipids and carbohydrates. Digestion of RNA with RNaseA can also help in reducing background noise. Humidity

and drying time can be assessed by examination of the chromosomes themselves. Chromosomes that look “shiny” or “hollow” will most likely give a poor or inconsistent hybridization pattern. This is indicative of high humidity and prolonged drying time. The effects of such variables can be minimized by preparation of the slides in an environmentally controlled unit in which both the temperature and humidity can be tightly controlled (Spurbeck et al., 1996).

Another highly variable parameter is the manner in which the slides themselves are stored. All protocols require that the slides be dehydrated after preparation and again prior to hybridization. One difference that arises is in the amount of time between preparation and the first dehydration. The length of time the slides “bake” at 37°C can vary anywhere from one day to one month. The slides are then dehydrated and either used immediately, stored in 100% ethanol at –20°C, or stored with Drierite or similar moisture absorbent material at –70°C for up to one year. Since water adversely affects the hybridization potential of the chromosomes, all of the above treatments are designed to prevent exposure to moisture.

The slides are then pre-treated prior to hybridization with the labeled probe. This involves exposure to RNase A followed by Proteinase K and/or Pepsin as indicated above. These treatments can be disregarded if there appears to be little evidence of surrounding cellular debris. Denaturation of the chromosomal DNA into single-stranded molecules is essential, however, and is accomplished by addition of 70% Formamide in 2XSSC (pH 7.0 – 7.5) to the slide followed by a coverslip and heating to 80°C for a predetermined period of time. The amount of time is dependent upon the species from which the chromosomes were prepared and the age of the slides, older slides generally needing slightly shorter incubation times. The coverslip is removed and the slides immediately immersed in ice cold 70% ethanol for  $\geq 3'$ . This is followed by a 90% and 100% ethanol dehydration series. The slides are air dried at room temperature, at which point they are ready for hybridization with the denatured probe.

The above protocol can also be adapted for adherent cells grown on chamber slides. This is sometimes preferable to trypsinization of the cells and preparation as a cell suspension. One application is the use of nucleic acid probes and fluorescent immunocytochemistry for the simultaneous localization of genomic sequences and particular proteins within the cell under growth conditions without perturbation of the cellular organization. This approach is being used more extensively as live cell imaging becomes an integral part of tumorigenesis studies.

Another variation involves the preparation of extended DNA fibers for ordering of probes with resolution in the range of 2 – 350 kb. There are at least three different methods

for obtaining these extended DNA/chromatin fibers. All three involve depletion of histones and extraction of genomic DNA from interphase nuclei on a microscope slide by high salt, alkaline or detergent treatment. In one protocol, the DNA forms halos around the nucleus in loops of 60 – 200 kb, the approximate distance between two matrix attachment sites in genomic DNA (Fig. 1D). Another method lyses the nuclei and the unattached DNA is allowed to stream down the microscope slide. The third approach involves treatment of intact cells with the topoisomerase II inhibitor m-AMSA to prevent chromatin condensation. Subsequent alkaline treatment of the nuclei permits the DNA to leak out of the nucleus. Chromatin extracted using the latter procedure results in more tangled DNA, however new techniques such as DNA combing (Monier et al., 1998) and optical mapping (Samad et al., 1995; Schwartz and Samad, 1997) have greatly improved this technique.

Other specimens utilized in situ hybridization protocols are formalin-fixed, paraffin-embedded tissue sections. These must first be treated to remove the wax and formalin. This is accomplished by heating the slides to 65°C for 2 hrs followed by incubation in xylene. As with the chromosome spreads, the slides are then run through an ethanol dehydration series, and as for the interphase slides, the cells are permeabilized by treatment with a 0.1% Triton X-100/4XSSC solution. The slides are then treated with a 1M sodium iso-thiocyanate (NaSCN) solution at 56°C to reverse fixative induced crosslinks between the DNA and protein. Pepsin treatment followed by the standard Formamide/2XSSC denaturation and ethanol series is performed prior to addition of the hybridization solution containing the denatured probe. Since the specimen preparation is typically performed the same day as the hybridization, storage of the slide after removal of the paraffin and formalin is not an issue.

The preparation of intact nuclei for interphase FISH is the same as for chromosome spreads from suspension or from tissue sections, depending on the source of the material. This includes treatment of the slide with RNase A, pepsin and formaldehyde as well as denaturation with formamide at elevated temperatures.

### **10.2.2. Preparation of DNA Probes**

DNA probes can be prepared from a number of different sources and their nature is dependent upon the type of the in situ hybridization being performed. The principles behind probe preparation are the same, however the exact protocol used will vary from source to source. The most common DNA probes consists of a fragment of genomic DNA or cDNA subcloned into a specific vector. This vector may take the form of a yeast (YAC), bacterial (BAC), plasmid (PAC) or human (HAC) artificial chromosome, a cosmid, plasmid

or phagemid. These types of probes are usually used for gene or translocation breakpoint mapping and chromosome identification.

The technique of spectral karyotyping (SKY), which will be discussed at greater length towards the end of this chapter, requires specially prepared probe sets. Briefly, this technique “paints” each chromosome with a unique combination of fluorochromes. Thus, a probe corresponding to the entire length of each chromosome must be prepared. The first step in this process involves the use of flow cytometry to separate the individual chromosomes of a particular species on the basis of size and relative nucleotide content (Carter et al., 1990; Gray et al., 1979). Each individual chromosome is then amplified by PCR using a degenerate oligonucleotide primer (Telenius et al., 1992; Telenius et al., 1992). This increases the amount of working material and serves as the labeling reaction by incorporating conjugated nucleotides during the PCR. Obviously great care must be taken not to cross-contaminate the sorted chromosomes prior to labeling.

Another source of probe is total genomic DNA isolated from cell lines or tissues. This type of probe is necessary for performing comparative genome hybridization (CGH) as well as another technique covered in Chapter 7 for the analysis of differential gene expression using DNA arrays. Isolation of genomic DNA from most cells or tissues (i.e. blood, cell lines, organs) involves an overnight incubation with Proteinase K and SDS followed by phenol:chloroform extraction and isopropanol precipitation. DNA can also be obtained from paraffin-embedded tissues, enabling one to retrospectively analyze archived tumor material. The only difference from the above protocols is removal of the formalin and paraffin prior to extraction of the DNA. This procedure is similar to that outlined in the previous section on metaphase preparation from paraffin-embedded, formalin-fixed tissue sections utilizing xylene and NaSCN.

Good quality DNA preparation is essential for obtaining a successful labeling reaction. Cross-contamination of probes during isolation must obviously be avoided, but this is not the only cause for trouble. Since the labeling reactions require the activity of enzymes, care must be taken to eliminate any contaminants which may be inhibitory to their activity. Many enzymes require the presence of divalent cations such as magnesium in order to function. This means that one must be careful of the concentration of chelating agents like EDTA or EGTA in the isolation steps. Some EDTA is acceptable as long as the final concentration in the labeling reaction is not too great. This can sometimes be overcome by addition of extra magnesium to the labeling reactions. Other potential inhibitors are proteinases or other non-specific proteins that may adversely affect the enzymes. The amount of protein in a DNA preparation can be gauged by calculation of the  $Abs_{260}:Abs_{280}$  ratio. This should normally be around 1.8 for DNA. This is generally the

easiest step in any hybridization reaction, but if done incorrectly can result in days of frustration and troubleshooting.

### **10.2.3. Labeling of DNA Probes**

Probes can be labeled with a number of different fluorescent and non-fluorescent conjugated nucleotide analogs. The type of label used depends upon the imaging equipment available, type of experiment, cost and personal preference. Probes containing nucleotides conjugated with a fluorescent molecule are said to be direct-labeled, whereas those containing nucleotides that are non-fluorescently labeled (i.e. with biotin, digoxigenin, dinitrophenol, etc.) are referred to as indirectly labeled. These latter probes must be detected using a second or third molecule containing the fluorescence as discussed in Section 2.5 of this chapter.

There are generally two different ways to label a probe for in situ hybridization. The first was briefly discussed in the previous section with regard to the chromosome-specific probes used in SKY. The conjugated nucleotides are incorporated directly into the PCR product. For SKY this usually involves the use of three fluorescence-conjugated and two non-fluorescence-conjugated dUTP analogs. This allows for the simultaneous hybridization and detection of probes that emit fluorescence in five distinct ranges of the spectrum. The importance of this will become apparent in later sections of this chapter dealing specifically with SKY. PCR can also be used to label genomic inserts with either insert-specific, vector-specific or degenerate oligonucleotide primers. DNA fragments smaller than 100kb can be efficiently labeled using random primed labeling (Feinberg and Vogelstein, 1983) and nick translation as described in the Introduction. Fluorochromes can also be chemically attached to nucleic acid probes.

### **10.2.4. Hybridization of Probe to Specimen**

This step of the procedures deviates very little from one technique to another. The desired amount of labeled probe is combined with an excess amount of competitor Cot I DNA (Landegent et al., 1987; Lichter et al., 1988; Pinkel et al., 1988). The purpose of the Cot I DNA is to suppress (by complimentary annealing) elements in the probe which are highly repetitive and dispersed throughout the genome. Such elements include Alu, LINE, SINE and B1 repeat sequences. Failure to eliminate these sequences from the probe will result in hybridization to sites on every chromosome, not just that region containing the unique sequence of interest. Cot I DNA can be eliminated, however, if the probe is known to be devoid of any repeat elements, which is often the case with cDNA probes. The combined DNA is precipitated, resuspended in a small volume of formamide/dextran

sulfate/SSC solution (referred to as hybridization buffer) and denatured by heating to 80°C. This is followed by either quick chilling (if no Cot I) or incubation at 37°C for at least one hour to allow the Cot I sequences to anneal, thereby making the repeat elements in the probe double stranded and preventing them from hybridizing to the sample.

The chromosomes on the slide are denatured by incubation in formamide at 80°C followed by a cold EtOH dehydration series. The probe is then allowed to anneal to the chromosomes at 37°C in a moist dark chamber. The amount of time required for hybridization depends on the complexity of the probe. The standard protocol is 18 – 24 hrs for unique sequence probes (i.e. genes, cDNA) and 40 – 72 hours for complex probe mixtures such as those used for SKY and CGH.

### **10.2.5. Detection and Visualization**

The detection stage of the protocol requires a number of increasingly stringent salt washes at 45°C to remove non-specifically bound probe. Bovine serum albumin (BSA) is used to non-specifically bind to the charged silica in the glass microscope slide thereby preventing a non-specific interaction between the slide and any other fluorescence-labeled molecules used in the detection process. Probes labeled with nucleotides directly conjugated to fluorescent molecules (i.e. FITC-dUTP, Rhod-dUTP, etc.) do not require blocking or further detection and can therefore be immediately analyzed. Indirect-labeled probes, however, require at least one more detection step. Bio-dUTP, for example, can be detected with either avidin conjugated to a fluorochrome (i.e. avidin-FITC) or with a fluorochrome-conjugated antibody directed against biotin (i.e. mouse-anti-biotin-FITC). One can add more detection levels in an attempt to increase the signal strength, however this usually results in an increase in the background level as well and therefore little improvement in the signal to noise ratio.

### **10.2.6. High Sensitivity Detection Procedures**

The smaller the actual target sequence in the genome, the shorter the probe becomes and thus fewer fluorescently-labeled nucleotides to provide a signal. A genomic target dispersed over a large physical distance can also result in decreased signal strength. An example of this is hybridization of a 6 kb cDNA clone. This size is generally sufficient for a nice signal, however if the gene contains multiple exons dispersed over perhaps 100 kb, the density of fluorescence is decreased and therefore more difficult to visualize. In order to circumvent this problem, multiple levels of immunological detection are sometimes used. As mentioned above, this often amplifies the background noise as well and is therefore not always an efficient means of detecting weak signals.

A newer method makes use of a well utilized enzymatic reaction involving peroxidase (van Gijlswijk et al., 1996) (van Gijlswijk et al., 1996) (Raap et al., 1995). This method was originally developed for ELISA (Bobrow and Litt, 1993) and has been adapted for immunohistochemistry (Adams, 1992) as well as for FISH (Raap et al., 1995) (van Gijlswijk et al., 1997). The probe is labeled in the usual manner by nick translation incorporation of a hapten-conjugated nucleotide (i.e. Bio-dUTP, Dig-dUTP). The first detection layer uses peroxidase conjugated to either avidin or an anti-hapten immunoglobulin molecule. These are then amplified through the use of a peroxidase substrate conjugated to either biotin or a fluorochrome. Upon cleavage of the substrate, the biotin or fluorochrome becomes deposited close to the site of the reaction. Fluorochromes can be immediately visualized while biotin deposits require detection with an avidin- or anti-biotin-fluorochrome conjugate. Because detection involves an enzymatic reaction, multiple substrates are cleaved and deposited, thereby amplifying the signal.

One problem with the original reported protocol was the loss of spatial resolution. This was due to the free migration of the cleaved substrate before its immobilization on the slide. Later modifications included polymeric substances to increase the viscosity of the reaction solution and thereby decrease the diffusion of the intermediate cleaved products (van Gijlswijk et al., 1996). The net result is an extremely sensitive amplification method for the detection of small or difficult to visualize FISH signals with only slightly sacrificing the spatial resolution of directly labeled probes. This technique is now available as a kit from NEN™ Life Science Products.

### **10.3. Applications of FISH**

Now that we have established an understanding of how FISH is performed, it is possible to explore the practical applications of this technique to biological problems. A review listing a number of applications came out almost ten years after the first publication describing non-isotopic in situ hybridization (Lichter and Ward, 1990). Although advances have been made and new techniques developed involving the use of FISH, the applications have not changed much. References cited in the following sections are not at all meant to be inclusive, but merely represent examples of the various applications.

#### **10.3.1. Gene Mapping**

The explosion in manuscripts involving the use of FISH came along as the human genome project began to take form. FISH proved to be a powerful method for mapping the many genes and genomic clones being isolated by a number of laboratories (Lichter et al., 1990). The initial goal was to use FISH to physically map sequences along each

chromosome at 1 Mb intervals (Bellanné-Chantelot et al., 1992; Lichter et al., 1990). This would allow gene hunters to use these markers as landmarks for linkage studies (Dixon et al., 1993; Lichter et al., 1992; Lichter et al., 1993). The use of Cot I suppression, as discussed in Section 2.4, was an immense improvement (Landegent et al., 1987; Lichter et al., 1988; Pinkel et al., 1988). Prior to this, the mapping of large clones resulted in hybridization to multiple loci and often gave a banding pattern to the chromosomes. This was due to the presence of highly repetitive sequences such as Alu, LINEs, SINEs, etc. in the probe and in those regions of the chromosome (heterochromatin) that are known to be relatively gene-poor. Suppression hybridization for review see (Jauch et al., 1990) effectively removed these sequences from the hybridization reaction thereby allowing only the single copy sequences to anneal to the chromosomes. The result was that FISH could be used to physically map the larger clones isolated from cosmid, BAC and YAC genomic libraries (Lengauer et al., 1991; Ried et al., 1990). Not only was FISH an effective means of mapping these clones, but it was also beneficial for determining whether they were pure or chimeric in nature. Chimeric clones are fusion products of DNA fragments from different genomic loci created during the generation of a recombinant library. This was often a problem with large YAC clones that contained from ~200 Kb to  $\geq 1$  Mb of genomic sequence.

FISH was, and continues to be, useful for physically mapping genes cloned by methods other than linkage where their chromosomal location is unknown (Gallagher et al., 1993; Otsu et al., 1993; Upender et al., 1994). This is especially true for identifying the location of homologous genes in different species (Chowdhary et al., 1998; Fronicke and Scherthan, 1997; O'Brien et al., 1997; Raudsepp et al., 1996; Wienberg et al., 1992). It is also a useful technique for the identification of pseudogenes or other genes in a gene family e.g. (Giordano et al., 1993) (Fig. 1A).

### **10.3.2. Somatic Hybrid Analysis**

Somatic cell hybrid lines have proved very useful for isolating and mapping disease genes in both mice and humans. It is important to determine the genomic contribution of the species of interest in these lines both during their derivation as well as periodically due to their unstable nature. This was originally accomplished through the employment of Hoechst 33258 or Giemsa stains. Mouse chromosomes could readily be distinguished in mouse-hamster hybrids by the intense Hoechst 33285 staining of the mouse centromeres (Hilwig and Gropp, 1972) while color differences with Giemsa staining was useful for the analysis of human-mouse hybrids (Bobrow and Cross, 1974). Giemsa banding of chromosomes was also used to identify inter-specific translocations (Kozak et al., 1977),

but the analysis required skilled knowledge of chromosome bands, was extremely time consuming and still was not sufficient for identification of more subtle rearrangements.

With the advent of FISH it became feasible to unambiguously identify species-specific chromosomes in inter-specific crosses. This was first accomplished by nick translation labeling the entire genome of a particular species and subsequent hybridization to metaphase spreads prepared from the hybrid cell line (Schardin et al., 1985) (Manuelidis, 1985) (Durnam et al., 1985) (Pinkel et al., 1988). In order to identify the origin of a particular chromosome, the cell line genomic DNA would be labeled and hybridized to normal metaphase chromosomes prepared from each of the species involved. In this manner it was possible to determine not only which chromosomes were being contributed from a given species, but how much and which regions of particular chromosomes were involved (Boyle et al., 1990) (Doucette-Stamm et al., 1991) (Fig. 1F). Another fluorescent technique was the performance of species-specific banding of the hybrid chromosomes using labeled *Alu* and L1 repetitive sequences as probes (Nelson et al., 1989) (Lichter et al., 1990).

### **10.3.3. Clinical and Cancer Cytogenetics**

Pre- and postnatal screening for cytogenetic abnormalities is extremely important as both a diagnostic as well as prognostic procedure. This is usually accomplished by Giemsa banding of metaphase chromosomes and karyotype analysis by skilled and certified cytogenetic technologists. Many chromosome aberrations, such as aneuploidy and translocations involving sufficiently large chromosome fragments, are readily identifiable through the use of these techniques. There are instances, however, where identification and determination of the exact defect is not possible by these methods. A prime example involves the identification of marker chromosomes. These are small chromosome fragments that usually contain some of the centromeric material necessary for segregation during cell division, but not enough chromatin to identify their origin by standard staining techniques (Sachs et al., 1987; Warburton, 1991). Identifying the chromosomal origin of these markers is clinically significant because not all extrachromosomal material has phenotypic consequences (Blennow et al., 1995).

Karyotype analysis of tumors is also very difficult by conventional banding techniques alone. This is due to poor morphology, the number of marker chromosomes, double minutes and complex chromosomal rearrangements often seen, especially in solid tumors (Heim and Mitelman, 1995). Karyotype aberrations increase in number as tumors progress from premalignant lesions to metastatic disease (Fig. 2A & B) (Ried et al., 1999). Identification of these changes is useful in determining the stage of the tumor for diagnostic

and prognostic purposes as well as following the course of regression and reappearance of tumors after treatment. Single chromosome paints proved useful in early endeavors using FISH as a complementary technique to Giemsa banding in order to further characterize tumor karyotypes (Carter, 1994). This method has been enhanced by the development of a new techniques known as spectral karyotyping (SKY) and m-FISH (Speicher et al., 1996) which allow for the simultaneous chromosome-specific painting of the human (Schröck et al., 1996) (Macville et al., 1997) and mouse (Liyanage et al., 1996) genomes. This technique and its many applications will be discussed in Section 4.2.

#### **10.3.4. Chromosome Evolution**

Comparative cytogenetics is the study of changes in chromosome number and composition within and between different species as a function of their evolutionary divergence from one another. Studies have been performed looking at the chromosomal evolution in humans (Ried et al., 1993; Wienberg et al., 1992), primates (Wienberg et al., 1990), great apes (Jauch et al., 1992) and gibbon (Koehler et al., 1995; Koehler et al., 1995; Müller et al., 1997). There is only one difference in chromosome morphology that distinguishes humans from chimpanzees (de Grouchy et al., 1972). Human chromosome 2 has been derived by the fusion of two acrocentric great ape chromosomes (chimpanzee chromosomes 12 & 13; gorilla and orangutan chromosomes 11 & 12). The only other difference was a translocation in gorilla between the chromosomes homologous to human chromosomes 5 & 17 (chimpanzee and orangutan chromosomes 4 & 19) (Jauch et al., 1992; Wienberg et al., 1990). A more striking pattern of chromosome evolution is seen when comparing the chromosomes of great apes to those of the gibbon. Only human chromosomes 11, 14, 20, X & Y hybridized to the entire length of a single gibbon chromosome. All other gibbon chromosomes were involved in interchromosomal rearrangements compared to humans and the great apes (Jauch et al., 1992; Wienberg et al., 1990) (Fig. 2C).

Similar studies have been performed to explain the karyotypic differences between the morphologically similar and closely related Indian and Chinese muntjacs (Yang et al., 1995; Yang et al., 1997; Yang et al., 1997). The Indian muntjac has the lowest chromosome number known in mammals, with a diploid genome of  $2n=6/7$ . The Chinese muntjac, however, has a karyotype of  $2n=46$ . It is curious from an evolutionary standpoint how the genomes of these two related species became so divergent. SKY analysis has also been applied to evolutionary studies and will improve the analysis of genomic relationships (Schröck et al., 1996).

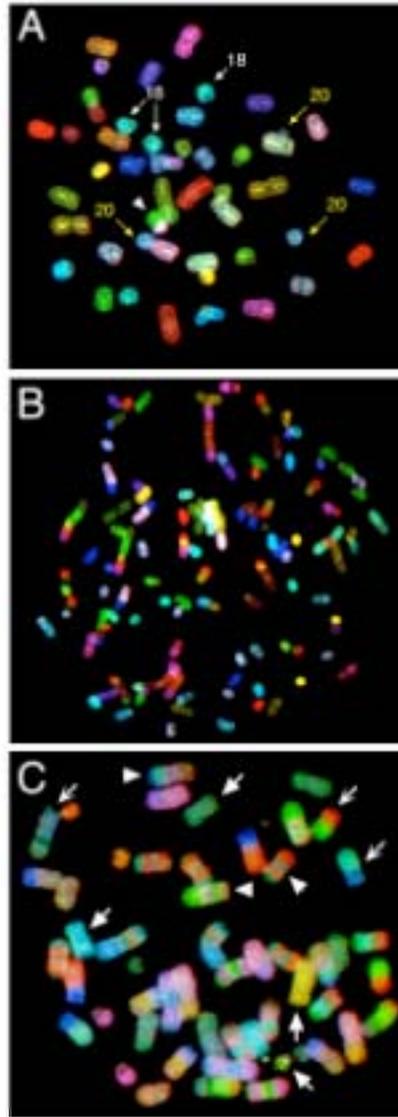


Figure 10.2:

- A. Immortalized cell line-CPDR-1 from a primary prostate cancer has a hyperdiploid karyotype with gains of chromosome 18 & 20 (arrows) and a t(Y;11) (arrowhead) (courtesy of M. Augustus).
- B. Highly rearranged chromosomes in a prostate cancer cell line (PC3) derived from a metastasis to the bone. The karyotype is predominantly hypertriploid (courtesy of M. Augustus).
- C. Differentially labeled human chromosome painting probes hybridized to metaphase chromosomes from the gibbon *Hylobates concolor*. Few chromosomes have not changed throughout evolution as indicated by their uniform hybridization color (arrows). Some chromosomes are simple rearrangements being composed of only two distinct human chromosomes (concave arrows) while the majority, however, have

become highly rearranged such that the gibbon chromosomes are composed of pieces from several different human chromosomes (arrowheads), or vice versa depending on your perspective (see Schröck et al., 1996).

#### **10.4. Karyotype Analysis**

The most commonly used method for whole genome analysis is Giemsa banding, in which the banding pattern of each chromosome is compared to its homologue and the standard banding pattern for chromosomes of that particular resolution. Although this remains an excellent technique for routine cytogenetic applications (i.e. prenatal diagnostics, reasons of infertility), analysis of more complex genomic rearrangements, such as those seen in tumorigenesis, requires great skill and even then subtle aberrations can be missed. The previous two sections have already discussed the use of FISH for analyzing the genomic complement of different individuals and species. Unfortunately, using standard FISH techniques it was only possible to analyze a few predetermined portions of the karyotype in any given hybridization. This was largely due to the limitations in the number of available fluorochromes that could be distinguished using conventional microscope hardware and imaging software. Recent advancements in fluorochromes and photodetection devices, however, have enabled the simultaneous hybridization and detection of probes labeled with multiple fluorochromes. The net result has been the development of combinatorial labeling and hybridization techniques known as spectral karyotyping (SKY) and m-FISH. Advancements in quantitative image analysis have themselves lead to the development of a new FISH application for whole genome analysis. This technique, referred to as comparative genomic hybridization (CGH), is a quantitative method for comparing the copy number of genomic regions between control and specimen samples. Each of these two methodologies alone provide significant advances in the way we analyze genomes, but their combination has resulted in a great leap forward in our knowledge and understanding of the chromosome aberrations specific not only to tumors of different origin, but also to specific developmental stages of tumorigenesis.

##### **10.4.1. Comparative Genome Hybridization**

The complex karyotypes found in solid tumors involve multiple translocations, chromosomal aneuploidy and the presence of small unidentifiable marker chromosomes. As a result, it is often impossible to know with certainty whether any genomic material has been gained or lost during the development of the tumor. A copy number change in individual genes of interest could be assessed by interphase or metaphase FISH, but until recently a comprehensive genome screen has not been possible. Comparative genome hybridization, or CGH, is a two-color FISH strategy that does not require growing cells

(Kallioniemi et al., 1992). As such, archived material is amenable to analysis at a later date. The technique involves nick translation labeling of total genomic DNA isolated from tumor and control samples with different fluorochromes. For illustrative purposes let us use rhodamine-dUTP (red fluorescence) for the control and fluorescein-dUTP (green fluorescence) for the tumor. These samples are pooled in a one-to-one ratio, combined with human CotI DNA to suppress repetitive elements and hybridized to normal human metaphase preparations.

The ratio of fluorescence intensities of the two fluorochromes is measured along the length of each chromosome (Fig. 3A). Sequences represented at the same copy number in the two genomes will hybridize as an equal ratio of the two colors giving a value of 1.0. Loss of chromosomal regions in the tumor will appear as regions with a ratio of red to green fluorescence greater than 1.0. Gain of material will result in greater hybridization of the tumor DNA (red) and a shift in the red to green ratio to values less than 1.0. The ability to make quantitative measurements requires fluorescence image acquisition using a charge-coupled device (CCD) camera and optical filters specific for the emission spectra of each fluorochrome. Special software packages compare the ratio of fluorescence intensities and output a karyotype with pseudocolored chromosomes representing gains and losses. A karyotype can also be produced which displays the red to green ratio along each chromosome as a histogram. This is important for determining the how many copies of a particular region have been gained or lost in the tumor sample.

Since the introduction of this technique in 1992, CGH has been applied to nearly all types of human cancers (Reviews (Ried et al., 1997) (Forozan et al., 1997) (Zitzelsberger et al., 1997)). This has resulted in the identification of tumor-specific and stage-specific aberrations that would have otherwise been unnoticed or more difficult to identify by conventional banding techniques. It has now become clear that what was originally thought to be a chromosome mess or a mere reflection of acquired secondary cytogenetic aberrations in solid tumors is actually a very specific gain or loss of genomic material. Determination of this pattern of copy number changes and identification of the genes found in these chromosomal regions will enable us to better understand the sequence of events on the molecular level which leads to the development of tumors in a tissue-specific manner. The ability to determine the particular developmental stage of a tumor based on its pattern of genomic gains and losses will have diagnostic and prognostic value. As we learn more about the different stages of tumorigenesis, we will be in a better position to tailor treatment to individual tumors based on their genetic profile.

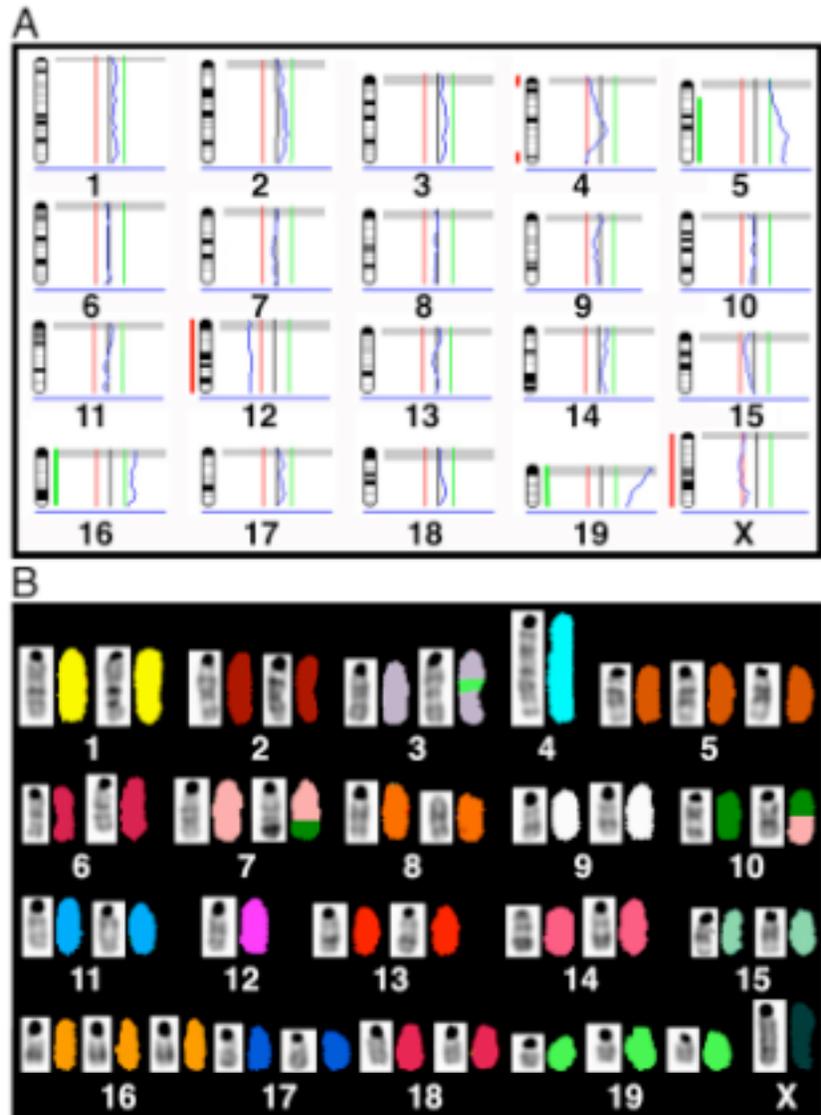


Figure 10.3:

- A. Comparative Genomic Hybridization (CGH) analysis of the metaphase in B. Illustrated is the G-banding pattern (ideogram) of each chromosome. To the left and right of the ideogram is indicated losses (thick red bar) and gains (thick green bar) of chromosome regions, respectively. To the far right of each ideogram are a series of lines. The red line indicates the loss threshold, the green line the gain threshold and the black middle line represents no change in the chromosome content. Superimposed on these threshold is a blue line representing the ratio of sample to control fluorescence (courtesy of C. Montagna).
- B. Spectral karyotype analysis (SKY) of a liver derived mouse tumor showing multiple chromosome rearrangements (Chr. 3, 4, 7 & 10), deletions (Chr. 12, X), gains (Chr. 5, 16 & 19) as well as a strain specific polymorphism (Chr. 8 - small centromere on right chromosome). Although a fragment of chromosome 19 has been inserted into the middle of chromosome 3, no material from chromosome 3 appears to be lost at the resolution of CGH (10.3.A). Translocation of chromosomes 4 to one another, however,

resulted in the loss of telomeric DNA from one chromosome and loss of centromeric DNA from the other. Reciprocal translocation between chromosomes 7 and 10 appears to be balanced since no material from either chromosome was gained or lost. Monosomy of chromosomes 12 and X as well as trisomy for chromosomes 5, 16 and 19 are all reflected in the CGH pattern of these chromosomes (courtesy of C. Montagna).

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#### **10.4.2. Spectral Karyotyping**

While CGH is a useful technique for the quantitation of genomic imbalances, it is often useful to also visualize balanced chromosomal translocations. As mentioned above, chromosome banding has limitations, especially in the analysis of tumor karyotypes. Individual chromosome paints can be used to look at chromosomes suspected of being involved in a rearrangement, but are not practical for obtaining an a priori assessment of genomic organization. Spectral karyotype analysis involves the simultaneous hybridization of all chromosome-specific painting probes to metaphase spreads (Liyanage et al., 1996; Schröck et al., 1996). This technique uses of five different fluorochromes. Each chromosome-specific library is PCR labeled using a degenerate oligonucleotide primer (DOP-PCR) and a unique combination of the five fluorochromes. For instance, chromosome 1 may be labeled with rhodamine (red fluorescence), chromosome 2 with fluorescein (green), chromosome 3 with Cy5 (blue), chromosome 4 with rhodamine and Cy5 (red and blue), chromosome 5 with fluorescein and Cy5 (green and blue), and chromosome 6 with rhodamine and fluorescein (red and green). By using a combinatorial labeling scheme, the number of probes that can be distinguished has increased from 3 to 6. Increasing the number of fluorescent molecules to 5 allows the distinction of up to 31 different chromosomes (reviewed in (Macville et al., 1997)).

The use of an interferometer attached to the CCD camera allows an analysis of the light with wavelengths between 400 and 800 nm captured at each pixel of the camera chip. A Fourier transformation is performed on the data which results in the generation of an emission spectra for each pixel. This is translated into an RGB display and the end result is a metaphase spread in which each chromosome has a distinct color. The chromosomes are automatically arranged into a karyotype based on their detected emission spectra and the combinatorial table, which indicates the labeling scheme used. Translocations are seen as the juxtaposition of two or more different colors on a single chromosome (Fig. 3B; i.e. chromosomes 7 & 10). This technique also enables the identification of marker chromosomes which either had no Giemsa bands or in which the banding pattern did not resemble that of any normal chromosome (Veldman et al., 1997) (Coleman et al., 1997). The combination of SKY and banding patterns (with either Giemsa or the fluorochrome

DAPI, which gives a reverse banding pattern) is a potent tool for the band identification of translocation breakpoints. FISH with individual clones in the region is useful for localizing the breakpoint to an individual clone. Designing primers and sequencing the region eventually leads to the identification of the exact breakpoint where the translocation occurred and the genomic reorganization of nearby candidate genes. Direct joining of two different gene segments can result in the formation of fusion proteins. These are seen in a wide variety of lymphoid tumors and are thought to be one of the causative agents responsible for tumorigenesis. The morphological similarity, and often poor quality, of mouse chromosomes makes SKY a particularly useful tool for the analysis of tumors found in murine models of human cancers (Coleman et al., 1997; Difilippantonio et al., 2000; Liyanage et al., 2000; Weaver et al., 1999; Xu et al., 1999).

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