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Cytogenetic mapping techniques: An approach to genome analysis

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ABSTRACT

Before the recent advances in molecular biology and the knowledge of the complete nucleotide sequence of several genomes, cytogenetic analysis provided the first information concerning the genome organization. The most important contribution of cytogenetics is related to the physical anchorage of genetic linkage maps in the chromosomes through the hybridization of DNA markers onto chromosomes. Cytogenetic approaches to studying chromosomes and their relationship to human disease have improved greatly over the past several decades. The cytogenetic mapping techniques of fluorescence in situ hybridization (FISH), which enables the direct chromosomal localization of labelled DNA probes and Comparative genomic hybridisation (CGH) is a technique that permits the detection of chromosomal copy number changes without the need for cell culturing. It provides a global overview of chromosomal gains and losses throughout the whole genome of a tumour. Another cytogenetic technique used to isolate populations of intact cells involved the process known as flow Cytometry which uses the fluorescent dye to label the cell population of interest. Practical applications of these techniques in genome analysis, chromosome mapping, analysis of somaclonal variations in tissue culture and many more have been presented.

KEYWORDS

Cytogenetic;
genome;
Fluorescence in situ hybridization;
Comparative Genomic hybridization;
Labelled DNA probes;
Flow cytometry;
Physical map.

INTRODUCTION

Cytogenetic is the study of chromosomes and their role in heredity. The methods that scientists use to analyse chromosomes, chromosome abnormalities associated with disease, the roles that chromosomes play in sex determination and changes in chromosomes during evolution[1,2]. A cytogenetic map is the visual appearance of a chromosome when stained and examined under a microscope. Particularly important are visually distinct regions, called light and dark bands, which give each of the chromosomes a unique appearance. This feature allows a person's chromosomes to be studied in a clinical test known as a karyotype, which allows scientists to look for chromosomal alterations[3]. These are based on the localization of features to the bands visible upon chromosomal staining. The simplest of the cytogenetic maps is the band map, which shows the positions of the bands themselves. The cytogenetic includes routine analysis of G-banded chromosomes, other cytogenetic banding techniques, as well as molecular

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cytogenetic such as fluorescent in-situ hybridization (FISH), comparative genomic hybridization (CGH) and flow Cytometry[4].

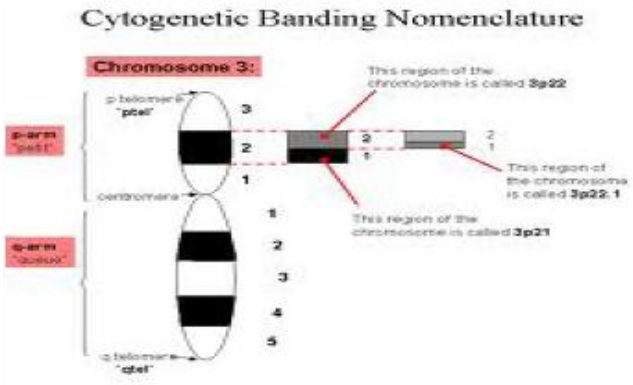

Figure 1 : Identification of cytogenetic map

TYPES OF MAPPING

1. Genomic Mapping: It is the creation of a genetic map assigning DNA fragments to chromosomes. It involves locating of a specific gene to particular region of a chromosome and determining the location of and relative distances between genes on the chromosome. The genetic linkage map shows the arrangement of genes and genetic markers along the chromosomes as calculated by the frequency with which they are inherited together[5]. The detailed genetic maps help locate the risk genes for a host of genetic diseases. Genetic maps of microbes enable researchers to harness the power of bacteria for producing energy from bio-fuels, reducing toxic waste, and developing environment-friendly products. The technique can also be used in organ transplants to achieve better matches between recipients and donors, thus minimizing the risks of complications and maximizing the use of donated healthy organs[6].

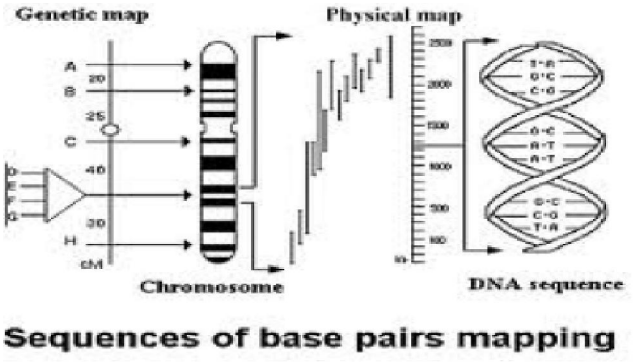

Figure 2 : Genome mapping

2. Physical Mapping: It is based directly on measurements of DNA structure. It uses a variety of methods to assign genes and DNA markers to particular locations along a chromosome, so the actual distances between the genes are known. It is useful in the large-scale isolation of genes using the positional cloning approach[7].
3. Restriction Mapping: It is the process of obtaining structural information on a piece of DNA by use of restriction enzymes such as endonucleases. A restriction map is a map of known restriction sites within a sequence of DNA. In molecular biology, restriction maps are used to determine the relationships between two different species at the molecular level. It is an easy way to compare DNA fragments without having any information of their nucleotide sequence[8].

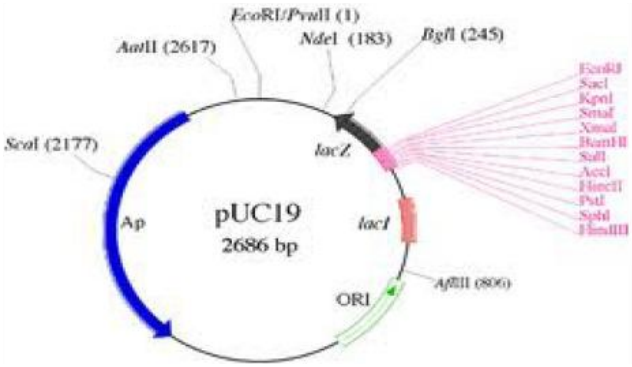

Figure 3 : Restriction map

4. Somatic Hybrid Mapping: Chromo some mapping through somatic cell hybridization is essentially based on fusion of human and mouse somatic cells. It involves the transfer of genetic material from the human cell to another type of cell in a culture[9].

CYTOGENETIC MAPPING METHODOLOGIES

Flow cytometry

Flow Cytometry measures multiple characteristics of individual particles flowing in single file in a stream of fluid. Light scattering at different angles can distinguish differences in size and internal complexity, whereas light emitted from fluorescently labelled antibodies can identify a wide array of cell surface and cytoplasmic antigens. This approach makes flow cytometry a powerful tool for detailed analysis of complex

populations in a short period of time[10]. Flow Cytometry is used for immunophenotyping of a variety of specimens, including whole blood, bone marrow, serous cavity fluids, cerebrospinal fluid, urine, and solid tissues. Characteristics that can be measured include cell size, cytoplasmic complexity, DNA or RNA content, and a wide range of membrane-bound and intracellular proteins[11].

Principle

Flow cytometry measures optical and fluorescence characteristics of single cells. Fluorescent dyes may bind or intercalate with different cellular components such as DNA or RNA. Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells. When labelled cells are passed by a light source, the fluorescent molecules are excited to a higher energy state. Upon returning to their resting states, the fluorochromes emit light energy at higher wavelengths. The use of multiple fluorochromes, each with similar excitation wavelengths and different

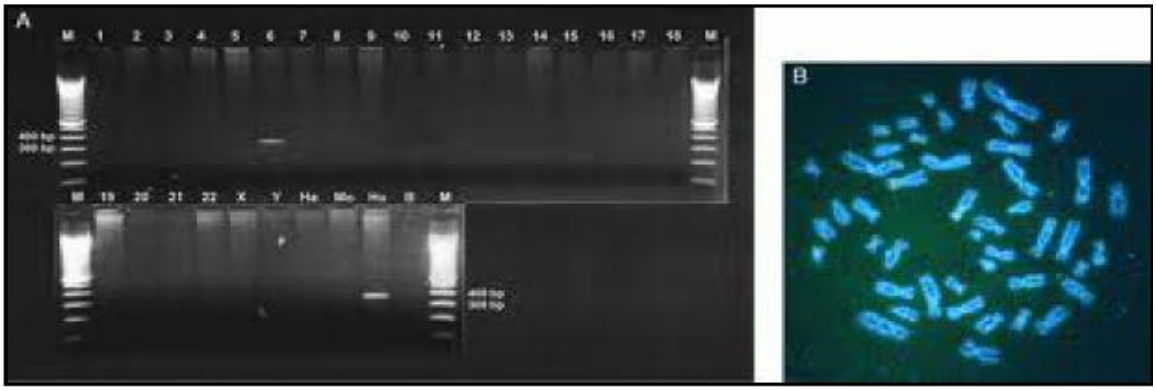

Figure 4 : Human/rodent somatic-cell hybrid mapping

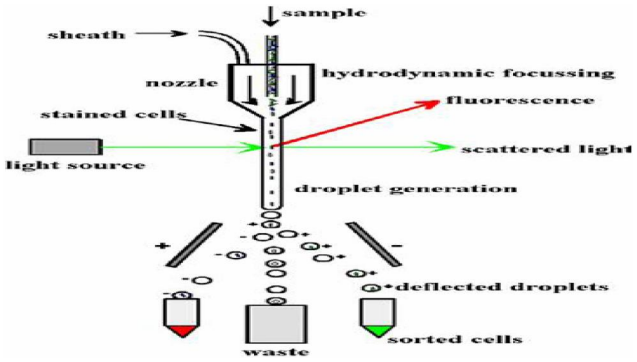

Figure 5 : Sorting of labelled chromosomes by flow cytometry

emission wavelengths allows several cell properties to be measured simultaneously[11,12].

Commonly used dyes include propidium iodide, phycoerythrin, and fluorescein. Although flow cytometry was initially used to isolate populations of intact cells, researchers adapted these techniques to isolate individual human chromosomes. Such techniques in-volve using mitotic cell suspensions and disrupting the cell membranes to release the condensed chrom-osomes that are labelled using two different types of fluorescent dyes. The first dye, called Hoechst 33269, binds to A-T base pairs, and the second dye, called chromomycin A, binds to G-C base pairs[14].

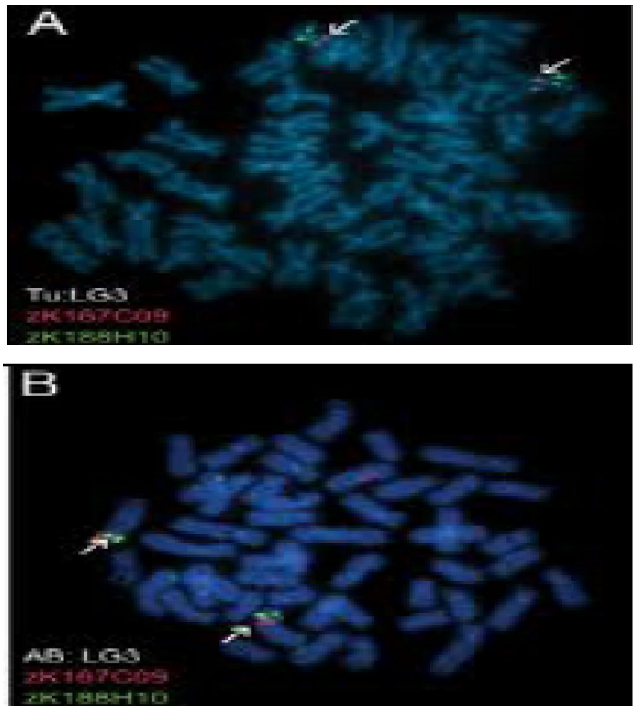

Figure 6 : Defining zebra fish genome using flow cytometry and cytogenetic mapping

The above figure represents that the zebra fish genome is first cytogenetically defined by first estimating the size of each linkage group chromosome using flow cyotmetry, followed by cytogenetic mapping of 575

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bacterial artificial chromosome clones onto metaphase chromosomes[13].

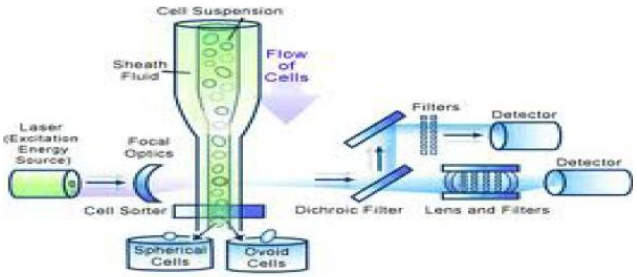

Figure 7 : Instrumentation of flow cytometer

Instrumentation of flow cytometry

Advantages of flow cytometry

This method is individual cell oriented which renders it quite suitable for quantification of cells from a heterogeneous cell sample.

It is quite a rapid method measurement being done at a rate of 1000 cells in a second.

Haemato cytometry is used in the study of blood cells, but it is too laborious and time consuming and accuracy of results is not at all reliable. These dis-advantages can be overcome by flow Cytometry[15].

Disadvantages of flow cytometry

1. Sensitivity : These are mostly designed to primarily analyse mammalian cells and so for bacteria, it does not function that well.
2. Portability : Commercially available flow cytometers are not portable and hence not suitable for field work.
3. Prior to use most of flow cytometers require adjustment.
4. Most of the flow cytometers are costly[15].

Applications of flow cytometry

1. DNA Content Analysis: The measurement of cellular DNA content by flow cytometry uses fluorescent dyes, such as propidium iodide, that intercalate into the DNA helical structure. The fluorescent signal is directly proportional to the amount of DNA in the nucleus and can identify gross gains or losses in DNA. Abnormal DNA content, also known as "DNA content aneuploidy", can be determined in a tumour cell population. Although conventional cytogenetic can detect smaller DNA content differences, flow cytometry allows more rapid analysis of a larger number of cells[20].
2. Genome size Analysis: Analytical information about the physical size of plant genomes and their state of replication is easily obtainable from flow cytometry. Knowing the number of base pairs in a genome is valuable for studies of new species. Flow cytometry provides a fast and accurate way to look at changes in genome size during evolution and differentiation. Establishment of ploidy and aneuploidy changes during tissue culture, and examination of intra- and inter-specific variation of DNA content can all be important in plant hybridization, breeding, and genetic manipulation programs. It provides an accurate method for determining the proportions of cells in G1, S and G2/M stages of the cell cycle. These data can be used to calculate cell cycle times, which are needed in studies of the genetics and control of this process, and are useful for analysis of aspects of crop growth and development[18,21].
3. Flow Cytometry analysis of DNA content: has been used together with several other FCM techniques to characterize and distinguish the two different mechanisms of cell death: apoptosis and necrosis. The most common marker of apoptotic cells in FCM is the decreased stain ability of their DNA with a variety of fluorochromes[20,22].
4. Application also includes high speed sorting for construction of chromosome specific libraries that establish feasibility for the Human Genome Program and the construction of chromosome specific fluorescent in situ hybridization for research and clinical applications[19].
5. Flow Cytometry also finds use in quantification of radiation induced chromosome damage, detection of numerical and structural aberrations associated with genetic diseases such as Down's syn-drome[16,17,18].

Florescence in-situ hybridization technique

Fluorescence in situ hybridization is a microbial method that allows for the detection of whole bacterial cells and analysis of chromosomes via the labelling of the specific nucleic acids with fluorescently labelled oligonucleotide probes. Basically the FISH method uses fluorescent molecules to vividly paint genes or chromosomes so that they can be detected and identified. First, short sequences of single-stranded DNA probes are prepared. These probes hybridize or bind to complementary nucleic acids because they are

labelled with fluorescent tags, allow the researchers to see the location of those sequences of DNA. The results are viewed, usually with a scanning laser microscope[23]. Unlike most other techniques used to study micro-organisms, which require the cells be actively dividing, FISH can also be performed on non-dividing cells, making it a highly versatile procedure. The FISH method involves mainly four steps: fixation, hybridization, washing and detection. In FISH, fluoresceinated chromosome probes are used for cytological analysis and cytogenetic studies, and to detect intratumoral heterogeneity. In genetics, FISH provides a physical mapping approach to detect hybridization of probes with metaphase chromosomes and with the less-condensed somatic interphase chromatin DNA probes may be applied to cell preparations on a slide[4].

There are two methods for multicolour FISH. The indirect method uses biotin, dioxigenin and dinitrophenol as reporter molecules. They are detected by fluorochrome-cojugated avidin or antibodies. Fluoro-chrome-labelled nucleotides are used for probe labelling in the direct method. The direct coupling of reporter molecules like fluorochromes to probes eliminates the need for immune cytochemical detection. Thus the direct method has two advantages over indirect methods, i.e. better resolution and speed.

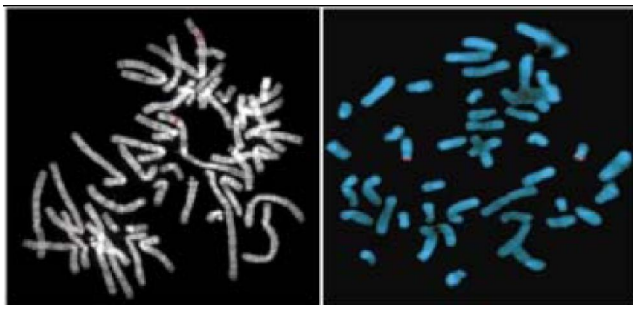

Figure 8 : Cytogenetic analysis of sequence integrated clones using FISH

Nederlof et al first described a method of detecting more than three targets[24].

Principle

The basic elements of FISH are a DNA probe and a target sequence. Before hybridization, the DNA probe is labelled by various means such as nick translation, random primed labelling and polymerase chain reaction. Two labelling strategies are used, for indirect labelling; probes are labelled with modified nucleotides that contain a hapten, whereas direct labelling uses nucleotides that have directly been modified to contain fluorophore. The labelled probe and the target DNA are denatured. Combining the denatured probe and the target allows the annealing of complementary DNA sequences. If the probe has been labelled indirectly, an extra step is required for visualization of non-fluorescent

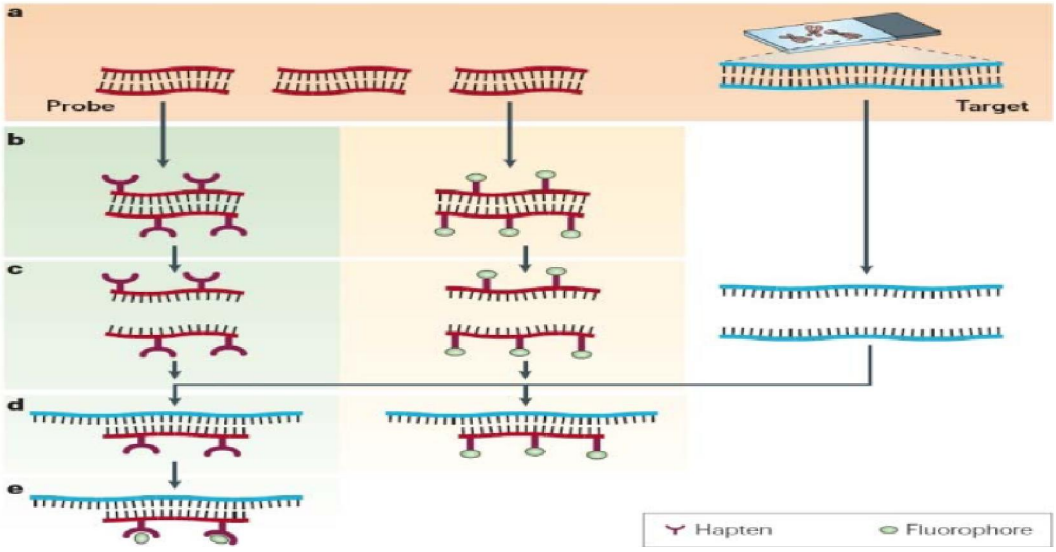

Figure 9 : Basic principle of FISH

hapten that uses a enzymatic or immunological detection system. Whereas FISH is faster with directly labelled probes, indirect labelling offers the advantage of signal amplification by using several layers of labelled antibodies and therefore produces a brighter signal[25].

Types of FISH probes

Scientists use three different types of FISH probes, each of which has different applications.

Locus specific probes hybridize to a particular region of a chromosome. This type of probe is useful

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when scientists have isolated a small portion of a gene and wish to determine which chromosome that gene is located on. They prepare a probe from piece of gene and observe which chromosome the probe hybridizes to[26].

Alphoid or centromeric repeat probes are generated from repetitive sequences found at the centromeres of chromosomes. Because each chromosome can be painted in a different colour, researchers use this technique to determine whether an individual has the correct number of chromosomes[23].

Whole chromosome probe are actually collection of smaller no. of probes, each of which hybridizes to a different sequence along the length of same chromosome. Using these libraries of probes, scientists are to paint an entire chromosome and generate a special type of karyotype. This full colour image of chromosome allows scientist to distinguish between the chromosomes based on their colours, rather than based on their dark and light banding patterns. These are useful for examining chromosomal abnormalities[23,26].

Advances in FISH technology

Multiplex-FISH and spectral karyotyping

One of the most appealing aspects of FISH technology is the ability to identify several regions or genes simultaneously, using different colours. In 1996, two groups independently reported a successful 24-colour karyotyping, termed multiplex-FISH (M-FISH)and spectral karyotyping (SKY),respectively. M-FISH and SKY allow painting of the entire chromosome complement in a single hybridization through labelling each chromosome with a different combination of fluorophore[27]. Images are collected with a fluorescence microscope that has filter sets for each fluorophore, and a combinatorial labelling algorithm allows separation and identification of all chromosomes, which are visualized in characteristic pseudo-colours. M-FISH and SKY differ only in the method used to discriminate the differentially labelled probes. SKY uses a dedicated imaging system that incorporates a cooled charge couple device (CCD camera) and Fourier transform spectrometry to analyse the spectral signature at each pixel of the image. M-FISH uses specific narrow band-pass fluorescence filter sets to reduce crosstalk and digital imaging equipment as part of a conventional epifluorescence microscope, with appropriate computer software[28]. The high efficiency of modern epifluorescence microscopes reduce typical exposure times limiting the effects of photo-bleaching. The achievement of 24-colour FISH-based karyotyping (M-FISH, SKY) has been one of the great successes of molecular cytogenetic in the past decade. The main applications for M-FISH have been the characterization of unbalanced translocations, complex chromosomal rearrangements and marker chromosomes in solid tumours, which are often distinguished by complex karyotypes. In common with other whole-chromosome painting methods, both M-FISH and SKY are not suitable for discriminating intra-chromosomal rearrangements such as duplications, deletions or inversions[29].

Applications of fluorescence in-situ hybridization

1. Detection of chromosomal aberrations: FISH and other *in situ* hybridization procedures are important in the clinical diagnosis of various chromosomal abnormalities, including deletions, duplications, and translocations. The figure shows how investigators used FISH together with standard karyotyping to analyse a patient translocation. The hybridization probe corresponded to a segment of chromosome 19 that was suspected to include the translocation breakpoint. Three areas of hybridization are apparent in the fluorescent image. One spot corresponds to the patient's normal copy of chromosome 19, and the other two spots correspond to the altered, or derived (der), versions of chromosomes 11 and 19 that were produced during the translocation. Thus, investigators were able to use the data both to narrow down the breakpoint region on chromosome 19 and to identify the second chromosome involved in the translocation[30,31].

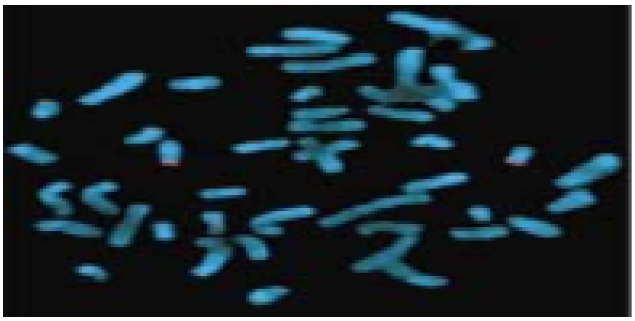

Figure 10 : Detection of translocation using FISH and karyotyping

2. Using FISH to analyse interphase chromosomes:

Since the introduction of FISH, cytogeneticists have been able to analyse interphase chromosomes as well as the metaphase chromosomes used in karyotypes. The figure shows an interphase nucleus from a patient with Charcot-Marie-Tooth disease. Here, the patient's cell has been hybridized with a red-labelled probe corresponding to a sequence within the duplicated region, along with a green probe corresponding to a sequence on chromosome 17 that lies outside of the duplicated region. From the two green signals, it is possible to locate two copies of chromosome 17 within the nucleus. One chromosome has the normal configuration, while the second contains the duplicated region, which is evident from two nearby red signals. Thus, two-colour interphase FISH provides a sensitive method

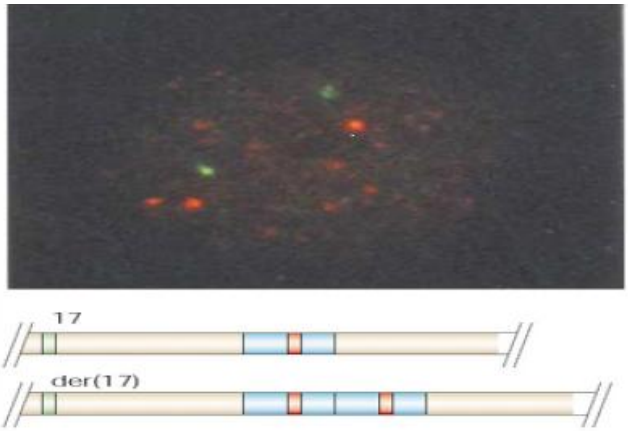

Figure 11: FISH used to detect chromosomal abnormalities in interphase nuclei

for analysing chromosome fusion events without the need for a prior cell culture[32].

3. Detection of Allien chromatin: In plant breeding programme, alien chromosome, chromosome segments, and genes can be identified and characterized by FISH. They can be visualized and counted in wide hybrids and amphidiploids. Subsequently, alien chromosomes can be followed through back crosses and recombinant lines. FISH has been used to identify partial amphidiploids derived from crosses of wheat with Thinopyrum intermedium and Lophopyrum elongatum with the resistance to wheat streak mosaic virus[33].
4. Analysis of Somaclonal Variations: These variations in tissue culture are considered as novel source of genetic variation for crop improvement. Using digoxigenin-labelled 5S rRNA and biotin labelled 18S-26rRNA, Lee et al compared the FISH patterns of regenerated autotetraploid plants with the diploid wild type in Allium cyaneum. The physical localization of rRNA genes in tetraploid species corresponded with that of diploid species. Thus, FISH method suggested that tetraploid regenerants originated from exact doubling of normal diploids[34].
5. Genome Analysis: Multi-colour FISH using total genomic DNA probes is a promising approach for simultaneously discriminating each genome in natural or artificial amphidiploids. This method is also a powerful tool for investigating genome homology between polyploidy species and their diploid progenitors. M-FISH has been used to differentiate three genomes in haploid wheat. Sometimes, adding large excess of unlabelled genomic blocking DNA from species not used as probe to the hybridizing solution improves differentiation between species of different genera e.g. genomes of two closely related species Hordeum bulbosum and H. vulgare were clearly distinguished[35].

Comparative genomic hybridization

One of the most significant developments for FISH in relation to genome-wide screening was the introduction of comparative genomic hybridization (CGH) in 1992. This modification of quantitative two colour fluorescence in situ hybridization utilizes genomic DNA from the sample under test to generate a map of DNA copy number changes in tumour genomes making it an ideal tool for analysing chromosomal imbalances in archived tumour material and for examining possible correlations between findings and tumour phenotypes[36,30]. In CGH, the genomic DNA from the specimen and control DNA extracted from an individual with a normal karyotype (46,XX or 46,XY) are differentially labelled with green and red fluorochromes respectively, mixed in equal amounts and co-hybridized to reference human metaphase chromosomes. The relative difference in DNA content between the normal and specimen DNA is represented by a difference in the green: red fluorescence ratios. The ratios of test to reference fluorescence along the chromosomes are quantified using digital image analysis[37].

One of the main advantages of CGH is its use as a discovery tool, as it requires no prior knowledge of the

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chromosome imbalance that is involved. CGH has also contributed significantly to the analysis of haematological malignancies in the identification of high-level amplifications, particularly in chronic lymphocytic

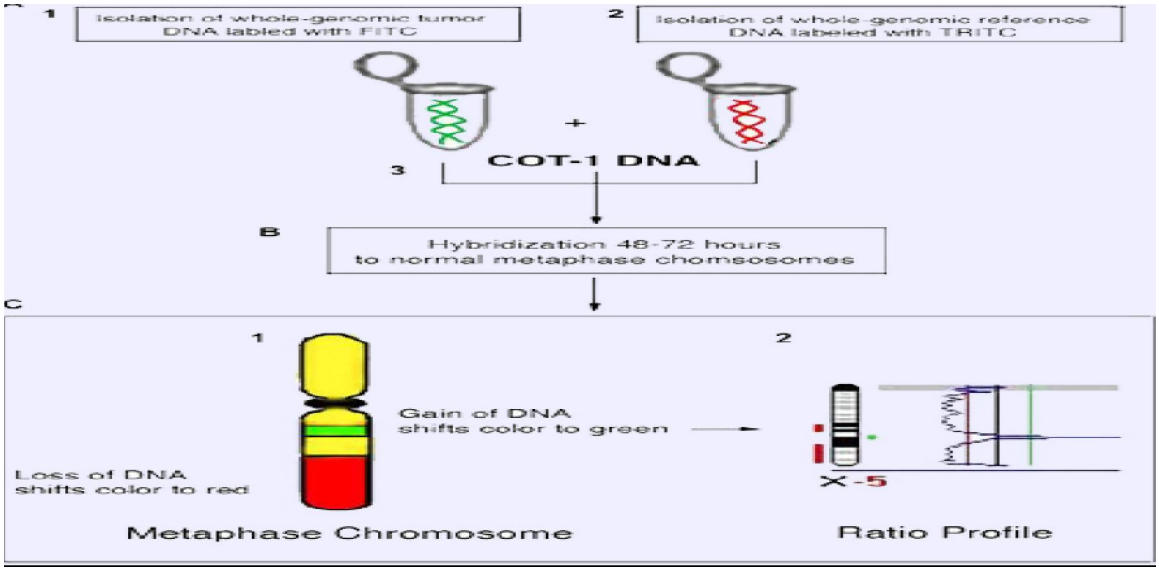

Figure 12 : Comparative genomic hybridization

leukaemia and non-Hodgkin lymphoma, and as an aid to classification schemes for the lymphomas.

Advantages of comparative genomic hybridization

1. Visualisation of deletions and duplications in very small DNA segments (which is of high importance as these can occur in birth defect syndromes and in cancer)
2. Searches of the whole genome without prior knowledge about the chromosomal aberration at hand
3. Analysis without the need for specific probes
4. The detection of the presence of amplified genes in cancer and maps their location
5. Unlike FISH, CGH is able to; Identify the chromosome with the aberration; identify the specific location from which the extra material originated; FISH is only able to identify the chromosome with the particular aberration.

Limitations of comparative genomic hybridization

1. For rearrangements that do not involve genomic imbalances, such as balanced chromosome translocations and inversions, the use of CGH is limited.
2. Whole-genome copy number changes (ploidy changes) cannot be detected.
3. CGH provides no information about the structural arrangements of chromosome segments that are involved in gains and losses.

Advances in comparative genomic hybridization

Array-comparative genomic hybridization

In array CGH, metaphase chromosomes are replaced as the target by large numbers of mapped clones that are spotted onto a standard glass slide greatly increasing the resolution of screening for genomic copy number gains and losses. In array CGH, the test and normal reference genomes, which are used as probes, are differentially labelled and co-hybridized to a microarray before being imaged. The relative fluorescence intensities are calculated for each mapped clone, with the resulting intensity ratio reflecting the DNA copy number difference[36]. The resolution of the analysis is restricted only by clone size and by the density of clones on the array. A further advantage is the ease with which array CGH can be automated for high-throughput applications. Array-CGH increased the ability to detect segmental genomic CNVs in patients with global developmental delay, mental retardation, autism, multiple congenital anomalies and dysmorphism, and is becoming a powerful tool in disease gene discovery and prenatal diagnostics[41] . The flexibility of array design has also allowed the development of specialized arrays for applications such as telomere screening or for specific diseases such as, B-cell leukaemia.

Principle

The principle of array CGH is derived from traditional CGH on metaphase spreads. In typical array CGH, the tested genomic DNA sample and a reference genomic DNA sample are labelled with different fluorescent dyes and co-hybridized to a solid surface, such as a microscope slide, onto which a large number of small DNA fragments of known chromosomal location are arrayed. These fragments are usually either 100–200 kb inserts of bacterial artificial chromosome (BAC) clones or, depending on the platform, 25–80 nucleotide-long oligomers[39]. A copy-number gain in the test sample (e.g., a micro duplication) for a region that is represented on the array will result in a relatively more-intense signal from its fluorescent dye, whereas a copy-number loss in the test sample (e.g., a micro deletion) will result in a relatively more-intense signal of the fluorescent dye of the reference sample.

CGH arrays that use large-insert genomic clones (such as BACs and P1 artificial chromosomes) are able to detect single-copy changes accurately and reliably. The use of BACs with known map positions allows direct correlation of DNA copy-number gains and losses with specific genomic sequence of known chromosomal locations. In many of the studies, array CGH identified abnormalities that were undetected by either conventional chromosome analysis or FISH[40].

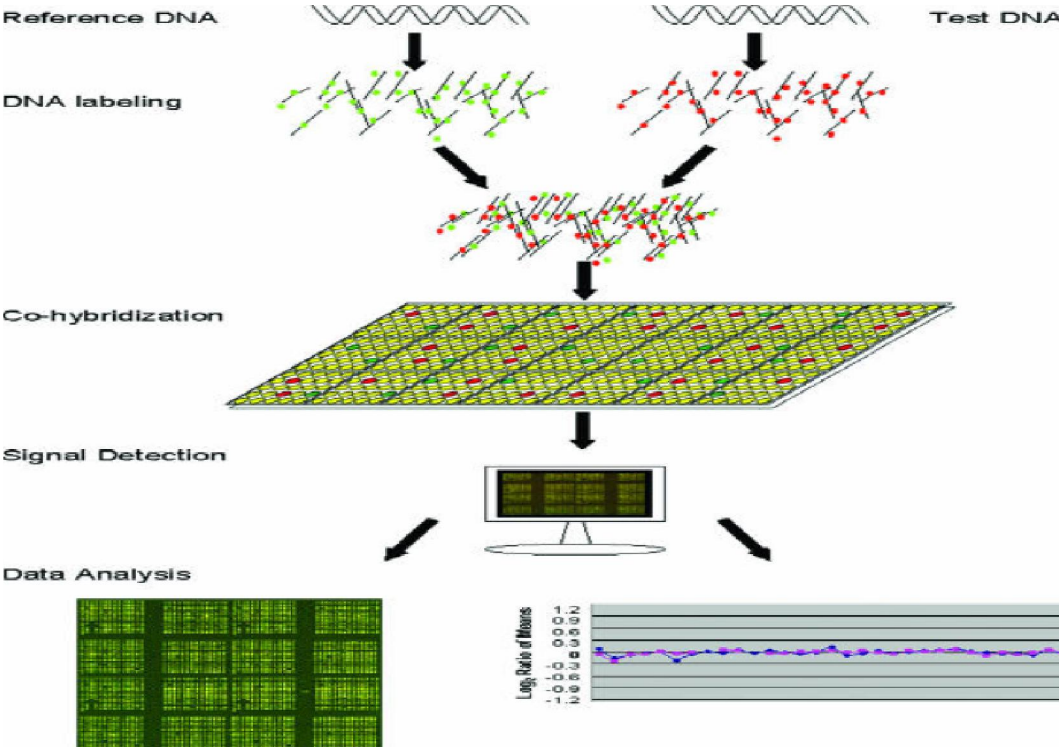

Figure 13 : Representation of Array-CGH

Research and diagnostic applications of Array-CGH

1. The use of array CGH in research has accelerated the pace of gene discovery in human genetics, deepened the understanding of genomic changes in cancer, and furthered the study of fundamental concepts related to chromosome conformation, DNA methylation, histone acetylation, gene silencing, replication timing, and many other basic mechanisms pertaining to DNA structure and function[42]
2. The high resolution afforded by array CGH has been used to define candidate regions for putative genes responsible for human genetic diseases. Vissers et al., hybridized cell lines from two individuals with CHARGE syndrome onto a genome-wide array with a 1-Mb resolution[43].
3. Array CGH has proven useful in providing DNA copy number profiles for various cancers. Many cancers are associated with multiple gains and losses of chromosomes and chromosomal segments. Many cancers are associated with

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multiple gains and losses of chromosomes and chromosomal segments. The difficulties associated with culturing and obtaining quality metaphases from most solid tumours, CGH approaches directly examine the DNA content and link any dosage changes to chromosome abnormalities[44].

4. The widespread use of array comparative genomic hybridization (CGH) for the diagnosis of genomic rearrangements in children with idiopathic mental retardation, developmental delay, and multiple congenital anomalies has spurred interest in applying array CGH technology to prenatal diagnosis. Women undergoing prenatal diagnosis were counselled and offered array-CGH in addition to routine chromosome analysis. Array-CGH was performed with DNA directly from amniotic fluid cells with whole genome amplification, on chorionic villus samples with amplification as necessary, and on cultured cells without amplification[45].

CONCLUSION

The advent of FISH in cytogenetic in has proved invaluable in both diagnostics and research. The power of its ability to identify specific genetic aberrations has propelled FISH-based techniques to the forefront of screening procedures for prenatal, paediatric and adult cases in a wide variety of cell types, including paraffin-embedded tissue, making FISH analysis data a useful tool in the decision of therapy to combat cancer. The near future of FISH lies in array-based technology, either with disease-specific CGH arrays that test for every known abnormality for a particular cancer; or disease-specific microarrays that display the mRNA expression levels of any oncogene and tumour suppressor gene. Thus, these cytogenetic methodologies with the knowledge acquired effectively directed towards the research and development of better treatment strategies to benefit the sufferers of diseases based on these genetic aberrations.

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