FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

The classical cytogenetics used trypsin-Giemsa or fluorescent banding pattern for identification and characterization of different chromosomal abnormalities such as polycentric chromosomes, ring chromosomes, or chromatid interchanges. Though chromosome banding techniques based on Giemsa staining revolutionized cytogenetic analysis, they did not become popular because of limited resolution involving only >3 Mb of DNA. Certain chromosomal aberrations such as reciprocal translocations and inversions were not easily recognizable with Giemsa stain. Besides that these techniques are very time consuming, and interpretation of karyotype is very cumbersome and uncertain.

In situ hybridization techniques initially developed by Joseph Gall and Mary Lou Pardue in 1960s and John et al. have proved to be powerful tools for determining the chromosomal location of hybridized nucleic acid. Soon after that fluorescent labels quickly replaced radioactive labels in hybridization probes because of their greater safety, stability, and ease of detection, leading to the development of Fluorescent in situ hybridization (FISH).

Early in situ studies used radioactive RNA or DNA probes that were labeled with ^{3}H or ^{135}I, and the sites of hybridization were detected by autoradiography. These techniques have been successfully applied to both animals and plants. RNA probes can be designed for any gene or any sequence within a gene for visualization of mRNA, long noncoding RNA and miRNA in tissues and cells. These probes, often derived from the fragments of DNA that were isolated, purified, and amplified for use in Human Genome Project, consist of about 20 oligonucleotide pairs and cover a space of 40–50 bp of target RNA. In 1982, a new method was described to localize DNA sequences hybridized *in situ* to chromosome. This method utilized a biotin-labeled analogue of thymidine (TTP) which could be incorporated enzymatically into DNA probes by nick translation. The sites of hybridization were detected either cytochemically by using avidin conjugated to horseradish peroxidase, or fluorometrically by using fluorescein-labeled antibodies. Compared to autoradiography this technique decreased the time required for detection, improved resolution, and gave less non-specific background and chemically stable hybridization probes. Besides that non-isotopic techniques have been developed using DNA probes labeled with amino acetyl fluorene (AAF), mercuration, and sulfonation, which are detected after hybridization by affinity reagents. Recently a very effective system has been described that uses digoxigenin-labeled nucleotides detected by antibodies carrying fluorescent or enzymatic tag. The non-isotopic labeling techniques have also been successfully applied for detection of highly repeated DNA sequences in plant chromosomes. The non-isotopic detection of low- or single-copy genes, however, has not been successful.

Chromosome painting – competitive hybridization using entire chromosome – specific libraries for chromosomes as probes and human genomic DNA as the competitor was one of the first applications of FISH (Fig. 1). It provided intense and specific fluorescent staining of human chromosome in metaphase spread and interphase nuclei. A translocation was first identified in human neoplasia leading to Philadelphia chromosome.

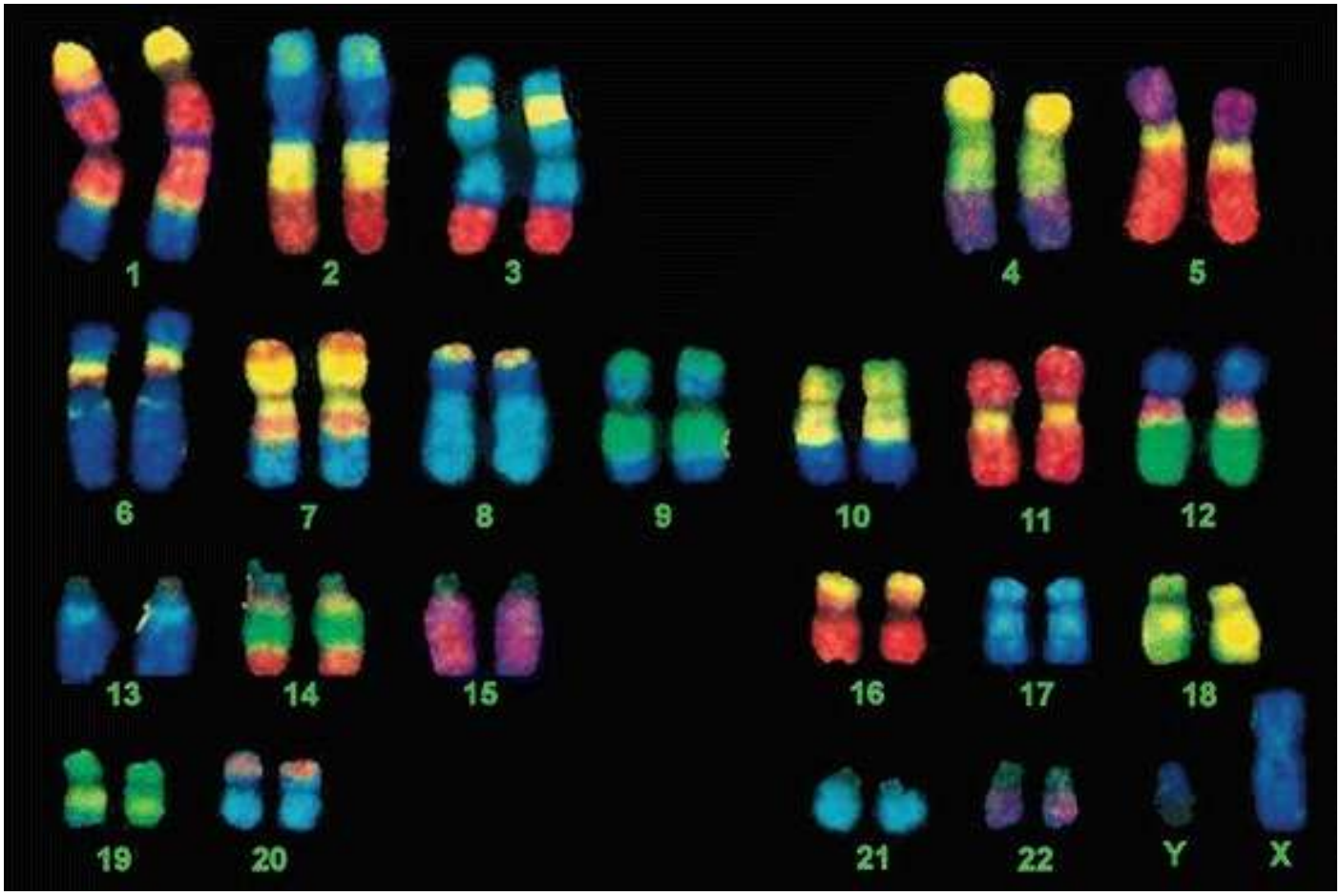

Fig.1 *Fluorescent in situ hybridization (FISH) identification of human chromosomes through chromosome painting. DNA probes specific to regions of particular chromosomes are attached to fluorescent markers and hybridized with a chromosome spread. The picture shows a computergenerated "false color" image, in which small variations in fluorescence wavelength among probes are enhanced as distinct primary colors. The combination of probes that hybridize to a particular chromosome produces a unique pattern for each chromosome. This makes it particularly easy to detect segmental deletions and translocations among chromosomes.*

Fluorescence in situ hybridization (FISH) began with the discovery that nucleic acids could be chemically modified to incorporate a hapten such as biotin or digoxigenin, which in turn could be detected with a fluorescently labeled reporter molecule such as avidin or anti-digoxigenin. Since then probe preparation and labeling techniques have been modified and simplified. Now nucleotides can be labeled with fluors directly and incorporated into FISH probes, eliminating the often laborious detection steps.

Principle Involved in FISH

The basic principle involved is hybridization of nuclear DNA of either interphase cells or of metaphase chromosomes affixed to a microscopic slide, with a nucleic acid probe. The probes are either labeled indirectly with a hapten or directly through incorporation of a fluorophore. The labeled probe and the target DNA are mixed together after denaturation, which allows annealing of complementary DNA sequences. In case the probe had been labeled indirectly, an extra step of enzymatic or immunological detection system will be required for visualization of the non-fluorescent hapten. Finally the signals are evaluated by fluorescence microscopy (Fig.2). The enzymatic detection system involves fluorochrome, which emits colored signals at the hybridization site. The immunological detection system is based on binding of antibodies to specific antigens, which is then demonstrated with a colored histochemical reaction visible by light microscope or fluorochromes with ultraviolet light.

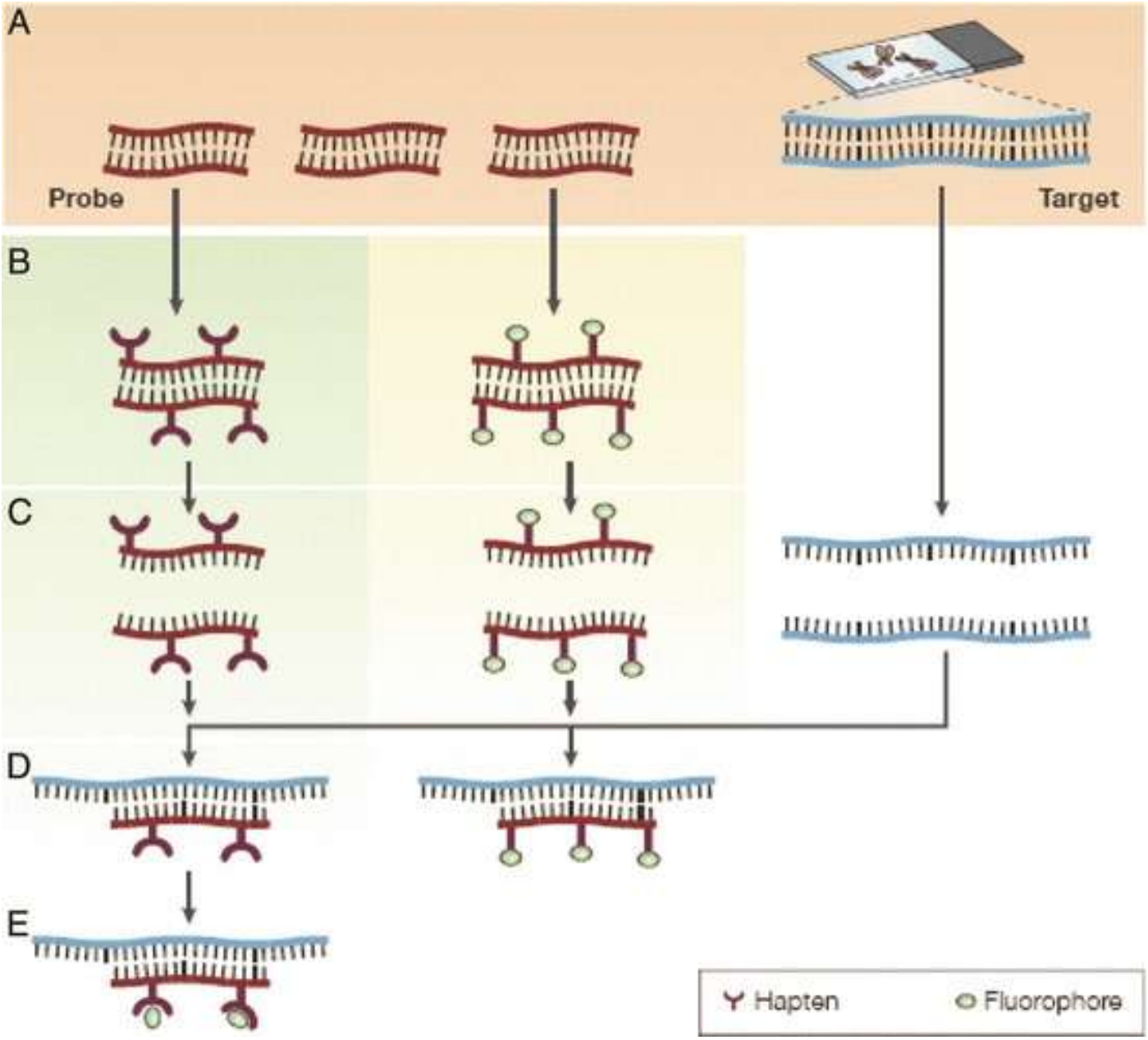

Fig.2 *The principles of fluorescence in situ hybridization. (**A**) The basic elements are a DNA probe and a target sequence. (**B**) Before hybridization, the DNA probe is labeled indirectly with a hapten (left panel) or directly labeled via the incorporation of a fluorophore (right panel). (**C**) The labeled probe and the target DNA are denatured to yield single-stranded DNA. (**D**) They are then combined, which allows the annealing of complementary DNA sequences. (**E**) If the probe has been labeled indirectly, an extra step is required for visualization of the nonfluorescent hapten that uses an enzymatic or immunological detection system. Finally, the signals are evaluated by fluorescence microscopy*

For direct detection, the most frequently used reporter molecules are fluorescein (fluorescein isothiocyanate, FITC), rhodamine, Texas Red, Cy2, Cy3, Cy5, and AMCA. For indirect detection method, the reporter molecules typically used are biotin, digoxigenin, and dinitrophenol.

Preparation of Probes:

One of the most important steps in FISH analysis is the choice of probe. A wide range of probes, extending from whole genomes to small cloned probes (1–10 kb), can be used. There are basically three types of probes, each with a different range of applications, whole chromosome painting probes, repetitive sequence probes, and locus specific probes, which are briefly described below.

1. Chromosome painting refers to the hybridization of fluorescently labeled chromosome-specific composite probe pools to cytological preparations. This enables visualization of individual

chromosomes in metaphase or interphase cells and the identification of chromosomal aberrations. The whole chromosome painting probes are complex DNA probes derived from a single type of chromosome that has been PCR amplified and labeled to generate a “paint” which homogeneously highlights the entire chromosome. With this probe, the cytologically visible structural and numerical chromosome rearrangement in metaphase becomes obvious. The chromosomal paint is, however, not helpful in the analysis of interphase cells. Whole chromosome painting is now available for every human chromosome, allowing the simultaneous painting of the entire genetic complement in 24 colors. This has led to the development of two independent FISH techniques – multicolor FISH (M-FISH) and spectral karyotyping (SKY) – which have important diagnostic and research application values.

2. Repetitive sequence probes hybridize to specific chromosomal regions or structures that contain short sequences, which are present in many thousands of copies. For example, pan-telomeric probes target the tandemly repeated (TTAGGG) sequences present in all human chromosomes ends. Centromeric probes target the α- and β-satellite sequences, flanking the centromeres of human chromosomes. Satellite DNA probes hybridize to multiple copies of the repeat sequences present at the centromeres, resulting in two very bright fluorescent signals in both metaphase and interphase diploid cells. These centromere-specific probes are useful in detection of monosomy, trisomy, and other aneuploidies in leukemias and solid tumors.

Applications of FISH

FISH has now become an essential tool for gene mapping and characterization of chromosome aberrations. Since the target DNA remains intact, unlike in molecular genetic analysis, information is obtained directly about the positions of probes in relation to chromosome bands or to other hybridized probes. Using differentially labeled probes, chromosome aberrations on particular chromosomes or chromosomal regions can be easily defined. The diseases that have been diagnosed using FISH include Prader-Willi syndrome, Angelman syndrome, 22q13 deletion syndrome, chronic myelogenous leukemia, acute lymphoblastic leukemia, Cri-du-Chat syndrome, velocardiofacial syndrome, and Down syndrome. The analysis of chromosomes 21, X, and Y can identify oligozoospermic individuals at risk.

In medicine, FISH can be used for diagnosis, evaluation of prognosis, and evaluation of remission of a disease such as cancer. FISH can be used to detect diseased cells more easily than standard cytogenetic methods. High-resolution FISH mapping and ordering of probes relative to one another can be performed on released chromatin fibers and is termed fiber-FISH. Fiber-FISH has a wide range of resolution (I kb–I Mb).

One of the major advantages of FISH over conventional molecular biology is the provision of molecular information in the context of cell morphology. Targeting nuclear RNA and the corresponding genes within cells or within a single cell or from a single allele can provide important information about gene expression, processing, and transport of transcripts in normal and mutant cells. The use of RNA FISH for studying the intracellular localization of RNA has increased over understanding of in situ physical characteristics of DNA transcription and transport of RNA transcripts. Similarly FISH can be used to examine many interesting biological questions about nuclear organization. Three-dimensional nuclear DNA FISH can provide high-resolution information about sub-chromosomal domains, gene position, and the relationship of genes and their transcripts in different cells and during different stages of the cell cycle. Accurate analysis of three-dimensional FISH is highly dependent on excellent quality confocal microscopy and image analysis procedures.

FISH technology also allows genome-wide screening of chromosomal gains and losses, which is comparative in in situ hybridization (CGH). It is based on the comparison of genomic DNA from two different genomes and identifies chromosomal gains and losses of one genome relative to the other. CGH is performed in normal chromosome metaphase spreads, which is a distinct advantage for studying tumor samples. The resolution of identifying chromosomal gains and losses on metaphase chromosomes is several Mbs. However, this technique has been modified to increase the resolution to several Kbs by the technique of matrix or array CGH, in which the targets are cloned DNA fragments immobilized on the glass surface. This allows detection of low copy number gains and losses and may be used diagnostically to identify microdeletions or amplifications affecting only one or two genes.

Cancer cytogenetics has benefitted greatly from FISH technology, and hence the clinical laboratories have benefitted from the technique, since it is rapid and can be performed on tissues (fresh frozen or formalin-fixed paraffin-embedded), touch preparations, cytospins, or cell cultures. Since it is usually difficult to get chromosome spread from tumor cells, the use of interphase FISH directly on tumor samples (biopsies, section, and archived paraffin-embedded material) enables the determination of chromosomal aberration without the need for interphase chromosomes preparations. Numerical chromosome aberrations, chromosome deletions, and translocations can all be identified in interphase nuclei providing important diagnostic and or prognostic information.

The advent of spectral dyes and imaging has made FISH more colorful and even more powerful. Using multiple probes simultaneously provides important additional information that can now be obtained for a single sample using multicolor FISH techniques. The techniques allow for both a genome-wide screen of aberrations and a gene or chromosomal regain-specific analyses of specific aberrations in chromosomes and can be adopted for use in the analysis of interphase nucleic. Similarly, genome-wide screen for mRNA expression differences or for genomic aberrations can be performed by microarray FISH, which is based on the comparative hybridization of two samples onto arrays that represent either specific sets of genes or the whole genome. The targets used come as oligonucleotides, cDNA, or genomic arrays.