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Fluorescent in-situ Hybridization - Some of its Applications in Clinical Cytogenetics

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ABSTRACT

Fluorescent in-situ hybridization (FISH) is becoming more and more relevant as an important future tool in prenatal and pre-implantation genetic diagnosis and cancer cytogenetics. This review describes the FISH technique as applied to whole chromosome spreads and interphase cells and discusses its applications in clinical cytogenetics. Information is presented on the various types of probes and the subsequent hybridization and detection procedures. The potential use of this novel FISH technique in the diagnosis of numerical and structural chromosomal aberrations in routine karyotyping for prenatal diagnosis, tumour cytogenetics and pre-implantation genetic diagnosis is outlined.

Keywords: fluorescent in-situ hybridization, principles, probes, applications in clinical cytogenetics

INTRODUCTION

In 1959, Ford linked the abnormal chromosome constitution of 45, X to the peculiar phenotype of Turner's syndrome. This led to an increased interest in the association between abnormal chromosome constitution and some clinical syndromes. The advent of the "banding" techniques in the late 1960s helped to refine the detection accuracy and provided for more and better information about cytogenetic make-up of cells and contributed significantly to the localisation of certain genes[1]. This phenotypic-karyotypic correlation was done on the basis of visual deletions, insertions, duplications etc. of DNA segments of various lengths. However, many instances are known in which, because of a parent carrying a balanced translocation, two or more offsprings suffer from a deficiency and/or excess of chromosomal material. Some familial chromosomal arrangements are beyond detection by our current cytogenetic methods, although remarkable results have been forthcoming with high resolution banding[2,3]. Similarly, paracentric inversions in a parent may not be evident except when carefully studied with high resolution banding, but can give rise to a variety of abnormalities in children. Base pair substitutions, however do not become relevant in clinical cytogenetics as the currently known congenital anomalies are strongly linked to known absence, addition or transposition of segments of DNA rather than specific genes. In 1969, Pardue and Gall[4] developed the technique of in-situ hybridization (ISH), which allowed the visualisation of the outcome of nucleic acid association reactions in morphologically well preserved metaphase or interphase cells using radioisotopes, thus bridging the gap between conventional cytogenetics and molecular genetics. However, in spite of the high sensitivity and wide applicability, their use has been limited to specific research laboratories only, due to the problems associated with radioactivity viz. limited shelf life, safety measures and the long time required for detection, making it time consuming for routine procedures.

The modification of nucleic acid probes with a stable non-radioactive label removes the major obstacles which hinder the general application of in-situ hybridization[5,6]. Furthermore, it opens new ways and opportunities of combining different labels on a single metaphase or nucleus in one experiment. Non-radioactive methods entailing labelling and detection using either enzymatic or fluorescent methods have replaced ISH with fluorescent in-situ hybridization or FISH.

With the advent of sophisticated fluorescent microscopes, filters, cameras and image analysis, FISH has steadily replaced the existing systems and is becoming widely used. As such, though in modern clinical cytogenetics, the banding techniques still remain the most accepted tool for detecting minor and major numerical and structural chromosomal re-arrangements, FISH is increasingly being used as a complementary tool to reduce the reporting time from 1 to 3 weeks, to a couple of days for a preliminary report in high risk cases. The main advantages of the FISH method are the distinct hybridization signals seen in metaphase as well as interphase nuclei which help in rapid diagnosis, thereby shortening reporting time as well as in confirmation of chromosomal anomalies in standard karyotyping for prenatal and pre-implantation genetic diagnosis and cancer cytogenetics.

Principles of the FISH technique

Fluorescent in-situ hybridization entails the deposition of fluorescent molecules in the nucleus at the sites of specific DNA sequences. Specific DNA or RNA sequences of choice are labelled with reporter molecules. These "probes" and the target chromosomes are denatured. Complementary

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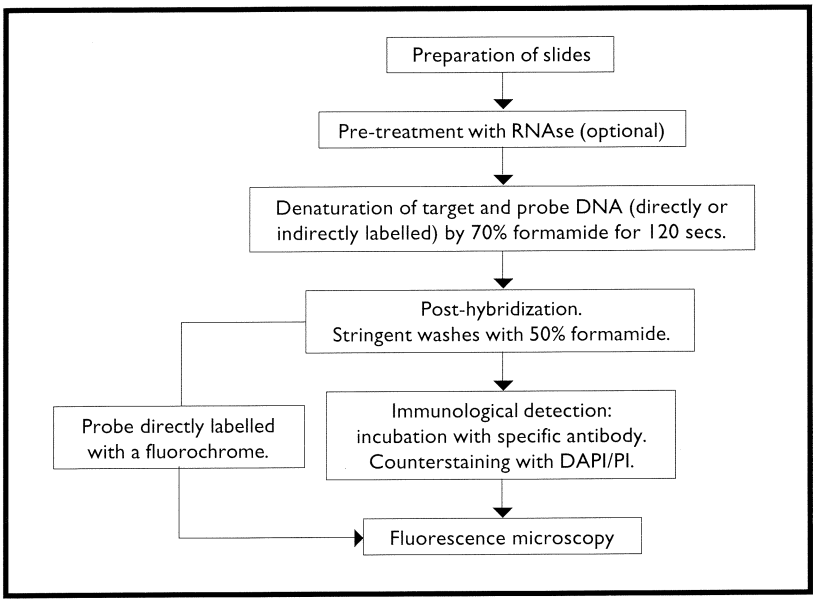

Fig 1 - Flow diagram for fluorescent in-situ hybridization

sequences in the probe and target are then allowed to reanneal. After washing and incubation with fluorescently labelled affinity reagents, a signal is made visible at the site of probe hybridization. Depending on how the probe is labelled, it can be detected directly or indirectly. For example, fluorochromes such as fluorescein can be directly coupled onto the probe and so visualised immediately under the fluorescence microscope after hybridization to the target DNA. Indirect procedures require that the modified probe be detected by immunocytochemical means (Fig 1). For example, biotinylated d-UTP incorporated in the probe is detected by the fluorescein avidin - anti-avidin system.

The basic technique comprises of preparation of the probe, preparation of the tissue, hybridization of probe to the tissue, stringent washes and visualisation of the probe.

Preparation of probe

The choice of the probe is the most crucial decision which has to be taken by the researcher and it depends on the application for which FISH is going to be used[7]. In clinical cytogenetics, since chromosomes are the main target, DNA probes from genomic DNA of a specific region are maximally used. RNA[8,9] probes also used in fluorescent in-situ hybridizations, find their major applications for detection of m-RNA expressions in cells and tissue material whereas oligonucleotide probes may be used if the sequence is known[10]. The most commonly used DNA probes are genomic fragments, which are either cloned into vectors or synthetically synthesised using various polymerase chain reaction (PCR) techniques. DNA probes produced by reverse transcriptase can be used where the gene product is known and the position on the chromosome has to be localised. It must be taken into account that in such cases, the probe (1-5kb) is contiguous, but the target is discontinuous and separated by intron sequences not present in the probe[11]. The different types of DNA probes, their synthesis and labelling are discussed below. A variety of probes are available commercially amongst others from Oncor (Gaithersburg, USA), Boehringer Mannheim (Germany) and Cambio (Cambridge, UK).

a) Types of DNA probes and their synthesis

i) Chromosome paints or whole chromosome probes

Individual whole chromosomes can be isolated using flow cytometry[12] or somatic cell hybrids[13]. These can then be cloned into suitable vectors by restriction enzymes and constructed into DNA libraries using yeast artificial chromosomes (YACs)[14] or cosmids[15,16]. Libraries can also be constructed using Alu[17] sequences coupled with PCR. Whole chromosome probes comprise of many different elements distributed more or less continuously over one chromosome so that the chromosome targeted by the probe appears continuously stained or painted (Fig 2a). Since these probes contain families of Alu repeat sequences that are on the chromosome from which the library was made and which are shared by other chromosomes to achieve the desired staining, these sequences are prevented from hybridising by addition of unlabelled blocking human genomic DNA to the probe mixture. This technique is called chromosomal in-situ suppression hybridization[18,19]. Translocations can be effectively detected, the only drawback being that not all libraries have equal specificity and sensitivity in detecting different chromosomal regions. The development of a degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) as a method of random amplification and labelling of DNA means that flow sorted chromosomes can be used directly instead of whole chromosome libraries and gives a superior and smooth coverage of the entire chromosome[20]. Another major advantage of using flow sorted chromosomes directly in this way is the facility for reverse chromosome painting where abnormal chromosomes can be sorted, labelled and painted back onto the normal chromosomes in order to determine their genomic derivation or vice versa[21,22].

ii) Locus specific tandemly repetitive sequence units are present in the centromere, heterochromatin and also on the arms of chromosomes of the human genome[23]. On most human chromosomes, some part of the repeated sequence is sufficiently different so that FISH with a probe to the variant region produces a signal that is intense and chromosome-specific (Figs 2b-d). Repeat sequence probes viz, alpha-satellite DNA probes, consisting of alphoid centromere repeats and the heterochromatic repeats are useful for tagging a particular chromosome of interest. These are tandemly repeated sequences, several hundred to thousand times in the centromeric regions about 10^6 to 10^8 bp in size. They belong mainly to the alpha satellite[24-26] or satellite III families. Alpha satellite sequences are comprised of 171 bp monomers, whereas satellite III are 5 bp monomers. The beta satellite probes (Oncor) hybridize to the heterochromatic regions on chromosome 1 and the acrocentric chromosomes 13, 14, 15, 21 and 22. As these are inherited in a Mendelian fashion, one can

Fig 2a - Painting probe for chromosome X showing 47, XXX in adult blood.

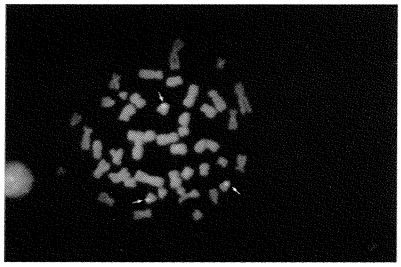

Fig 2b - Chromosome 18 alpha satellite probe showing trisomy 18 in cultured amniotic fluid cells.

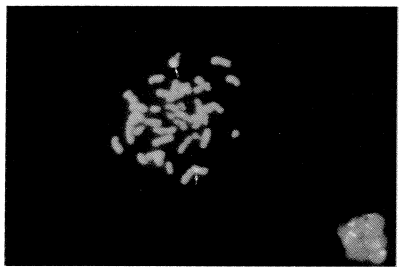

Fig 2c - Chromosome X alpha satellite probe showing a ring X along with a normal X in adult lymphocyte culture.

Fig 2d - 13/21 alpha satellite probe showing trisomy 21 in cultured fetal lymphocytes.

Fig 2 - Fluorescent in-situ hybridization using biotinylated probes on metaphase chromosomes which are counterstained with propidium iodide.

track parental lineage of a particular chromosome. Chromosome-specific repeat sequence probes have now been isolated and cloned for all human chromosomes and are commercially available. They are particularly useful for tagging a specific chromosome of interest, rapid sexing or scoring for chromosomal aneuploidy.

iii) Chromosome segment specific DNA probes

These probes can be obtained by chromosome microtechnology ie. mechanical microdissection or with a laser coupled with microamplification by microcloning[27] or PCR. The initial sample size for this technique is very small and so handling of these samples has to be done with great precision eg. standard cloning procedures start with 0.1μg to 1μg DNA while the DNA contained in 100 chromosome segments is approximately 3 picograms. Similarly, PCR amplification with single unique or Alu primers starts with 10 nanograms as compared to 30 femtograms for the DNA of a single chromosome segment[28]. PCR based approaches are sensitive, can generate larger number of libraries with a small number of chromosome segments and can be used to produce large quantities of probes from any part of the genome. These libraries can then be screened to isolate desired sequences on specific segments of the chromosome. Unique sequence probes and single copy probes[29,30] are the most powerful approach to structural aberrations viz, gene deletion or amplification[31] in both metaphase and interphase cells. Once the important loci in a particular genetic disease have been identified, they can be studied using FISH with probes to this region. Probes ranging from 15 - 50 kb of DNA sequences cloned into large insert phages, cosmids or YACs (50-500kb) have proved useful as specific locus probes[32,33]. The minimum size limit for reliable detection is around 3-5 kb, although probes under 1 kb[34] have also been detected.

b. Labelling of the probe

This entails the introduction of a reporter group ie. a detectable molecule into the probe. Based on the type of probe labelling, there are two types of FISH systems. In the direct method, the reporter is bound directly to the probe so that the probe-target hybrids can be visualised under a microscope immediately after the hybridization reaction[35] is achieved by the enzymatic introduction of fluorochrome labelled nucleotides. Indirect procedures require the probe to contain an enzymatically or chemically introduced reporter molecule, which is linked indirectly through an additional interaction between the modification group of the probe and a universal indicator molecule which specifically recognises and binds to the modified probe. The modification groups may be a vitamin (eg. biotin[36]), various haptens (digoxigenin DIG[37], fluorescein[38], dinitrphenol DNP[39], acetylaminofluorine[40] or heavy metal ions (mercury[41]). The most important indirect non-radioactive systems are the digoxigenin, biotin and DNP systems. The DIG system uses DIG specific sheep antibodies and anti-sheep antibodies coupled to a fluorescent dye. The biotin system uses avidin-anti-avidin coupled to a fluorescent dye, whereas the DNP system uses DNP specific rat antibodies and anti-rat antibody coupled to a fluorescent dye. The commonly used immunofluorophores with good spectral separation properties use fluorescein isothiocyanate (FITC), rhodamine, aminomethyl coumarin acetic acid (AMCA), Texas red and the cyanine dyes[42].

Introduction of the label can be done by random primed labelling, nick translation reaction and modified PCR techniques. Random primed labelling entails the use of the Klenow fragment of *E coli* DNA polymerase, random primer made of an oligodeoxyribonucleotide hexamer mixture, a hapten modified and a non-modified dNTP mixture. DNA to be labelled is linearised and treated to the above mixture for a complementary strand to be synthesised[43]. Nick translation involves the simultaneous action of DNAse I and DNA polymerase, whereby nicks are produced in each DNA strand and during re-synthesis, unlabelled nucleotides are replaced by labelled ones. Microdissected chromosome regions have also been successfully labelled using PCR with degenerate oligonucleotide primers and used as paints[44].

Preparation of tissue

Standard cytogenetic samples of metaphase spreads or interphase nuclei are fixed to allow access of a probe to single stranded DNA while maintaining the morphology. Fresh amniotic fluid cells too are treated with potassium chloride (0.075M) and fixative (methanol:glacial acetic acid:3:1) before use. RNAse treatment before applying FISH is helpful to reduce non-specific background signals and improves access of the probe through the protein matrix. Paraffin embedded sections must be de-waxed and protease digested before being probed.

Hybridization and post-hybridization washes

For in-situ hybridization to chromosomal DNA, the DNA target and probe must be denatured. This can be achieved by extremes of heat or the use of alkaline pH. In general, such treatments may lead to loss of morphology, so a suitable compromise must be found between the two. For heat denaturation, the probe and target chromosomal DNA may be denatured simultaneously by applying the probe to the slide, covering with a coverslip and treating at 80°C for 2 - 10 min. For competitive chromosome in-situ suppression hybridization (chromosome painting), the slides and probes are denatured separately.

Labelled probes can hybridize non-specifically to sequences which bear homology but are not entirely homologous to the probe sequences. Such hybrids are less stable than perfectly matched hybrids. They can be dissociated by manipulating formamide and salt concentrations as well as the temperature. Often a wash of 50% formamide/ 2XSSC will suffice.

Visualisation of the probe

Fluorescent DNA counterstaining is mostly performed with red propidium iodide (PI) or 4'-6-diamidino-2-phenylindole (DAPI), with the addition of an anti-fading reagent viz 1,4-diazobicyclo-(2,2,2) Octane (DABCO). Using standard fluorescence microscopes with suitable filter sets, repetitive sequences can be easily visualised. However, high quality microscopes are essential for single copy sequence detection. The confocal laser scanning microscope can be used, which utilises two laser lines operating at wavelengths suitable for FITC and PI or Texas red. Images are captured separately and the two digital images are merged. The restriction to using only two wavelengths can be overcome with the use of multiple band pass filters in conjunction with a charge coupled device (CCD) camera, which enables images to be collected with high resolution at whichever wavelength it is exposed to. In "multicolour FISH", the simultaneous visualisation of upto seven different probes labelled combinatorially has been described and more recently, the entire human karyotype has been visualised by combinatorial multi-fluor FISH, opening up unlimited scope for use of this technique[45].

Applications

Detection of numerical rearrangements

Prenatal diagnosis

Trisomies of the chromosomes 13, 18, 21 (Fig 3a) account for the majority of chromosomal abnormalities of diagnostic relevance in prenatal clinics. Some sex-linked disorders require the necessity of prenatal diagnosis of fetal sex. Repetitive sequences for the centromeric regions of 13, 18, 21, and X and for the long arm of Y produce distinct signals in metaphase as well as interphase cells[46-48]. Whole chromosome probes for nearly all the human chromosomes are commercially available. Oncor, Boehringer Mannheim (Germany), Cytocell (UK) and Cambio (UK) are some of the commercial manufacturers for centromeric, some loci specific and painting probes. This technique, when carried out on uncultured amniocytes results in a 24-hour diagnosis for the detection of a specific chromosomal abnormality in comparison to the 2-3 weeks for the conventional culture method. Use of multicolour FISH can facilitate diagnosis of different chromosomes on the same sample[49,50]. This is a very significant application most appropriate for prenatal diagnosis. It overcomes the significant drawbacks of metaphase analysis which requires cells to be cultured, which is labour intensive and time-consuming. Using the probes for the most commonly occurring aneuploidies viz 13,18, 21 and the sex chromosomes X and Y, FISH can be used as prescreen for detection of aneuploidies and the sex of the fetus in uncultured amniocytes (Fig 3b). However, all cells in the population may not show the trisomic signal due to chance overlap of the hybridization domains. Also, mosaicism is not reliably detected. Hence presently, FISH can only complement routine banded cytogenetics.

When compared to amniocentesis and chorion villus biopsy, both of which are invasive techniques, prenatal detection of chromosomal anomalies in fetal cells in the maternal peripheral circulation is a relatively non-invasive technique. With the use of a fluorescence activated cell sorter, nucleated erythrocytes, which are fetal in origin, can be separated and probed with FISH for aneuploidies of chromosomes of interest, thereby eliminating a lot of laborious techniques previously used[51,52].

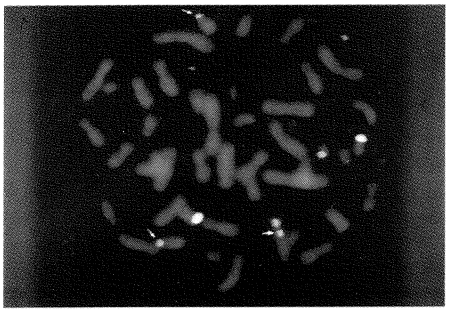

Fig 3a - Cultured fetal lymphocytes showing trisomy 13 in metaphase.

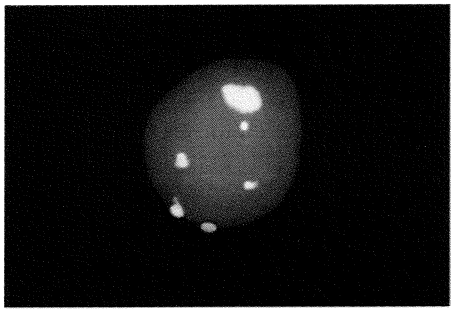

Fig 3b (i) - Uncultured amniocyte (interphase) showing two signals each for chromosomes 13, 21 and 18.

Fig 3b (ii) - Uncultured amniocyte showing one signal each for X and Y.

Fig 3 - Dual labelling, chromosome 13/21 labelled with digoxygenin-rhodamine and chromosome 18 labelled with biotin-fluorescein. Chromosome X is labelled with biotin-fluorescein and Y with digoxygenin-rhodamine. Counterstaining is done with DAPI.

Cancer cytogenetics

Chromosome analysis by classic cytogenetics is made more difficult in cancer patients due to the presence of marker chromosomes and the occurrence of multiple aberrations per cell. In addition, karyotyping can only be applied to cells that can be stimulated into mitosis and reliably banded. This is a significant limitation, especially in solid tumours. In breast cancer, for example, many of the cells that proliferate in culture are near diploid, even when the tumour appears highly aneuploid by DNA content analysis. With the help of FISH, it is possible to detect the number of copies of a specific chromosome per cell and to date, analysis of aneuploidy of cancers of the breast[53], bladder[54], testis[55], brain[56], stomach[57], colon[58] and in leukaemia[59] have been described. Multicolour FISH of the entire set of human chromosomes on a single metaphase plate will be particularly useful in cancer cytogenetics.

Genetic diagnosis of preimplantation embryos

The introduction of the FISH technique has proven invaluable in preimplantation genetic diagnosis. Double target in-situ hybridisation has been performed with X & Y specific probes on embryos to check sex[60,61], as well as with autosomal probes to detect specific chromosomal aneuploidy and mosaicism[62]. A further achievement has been the use of PCR and FISH on cleavage and blastocyst stages of cultured human pre-implantation embryos[63,64]. The use of multicolour FISH has recently contributed to the confirmation of the incidence of numerical rearrangements in abnormal human embryos generated in IVF programmes and provided explanations to some of the causes of IVF failures in some patients[65]. Centromeric probes allow precise counting of chromosomes from blastomeres and as such, give more reliable and accurate information on numerical rearrangements compared to the conventional method of fixation of embryos[66].

Structural aberrations

With the use of painting probes, translocations in cultured leucocytes and amniocytes can be easily detected, but more so in metaphase cells[67,69]. Used in conjunction with routine Giemsa banding, translocations involving a specific chromosome can be ascertained. The use of FISH with YAC clones in particular is a valuable approach to positional cloning. Once a YAC is identifed as crossing the breakpoint, it can be subcloned as a first step to cloning the breakpoint. This is of special use in cancer cytogenetics, as the number and morphology of chromosomes is uncertain[70-72]. Detection of translocations can be made even more accurate by hybridizing with probes targeted to the locus of interest. For example, hybridization with a digoxigenin labelled probe for a 15 kb region of the BCR gene on chromosome 22 (proximal to the CML breakpoint region) and a biotin labelled probe for a 35 kb region of the ABL gene on chromosome 9 (distal to the CML breakpoint region) enables detection of the BCR-ABL fusion event associated with chronic myeloid leukemia[73]. Analysis of reciprocal translocations by chromosome painting has some limitations. Not all libraries have equal specificity and sensitivity in detecting different chromosomal regions. These should be taken into consideration when selecting a library. However, with the refinement of techniques, this problem will soon be minimised.

The principle of reverse chromosome painting, whereby abnormal chromosomes are sorted, labelled and painted back on to normal chromosomes to determine their genomic derivation, has been used with genomic tumour DNA as probe onto normal chromosomes to reveal positions of amplified DNA sequences[74].

Detections of deletions or inactivation of tumour suppressor genes such as p53 and Rb1 or amplification of oncogenes such as erB-2 and c-myc is found frequently in advanced human solid tumours[75]. With the use of comparative genome hybridisation (CGH) entire genomes can be surveyed for DNA sequence-copy number variation. In CGH the relative intensities of tumour and normal reference DNAs after hybridization to normal metaphase chromosomes is used to reveal and map regions of increased DNA sequence copy number[76].

Hence the future prospects for FISH are very vast. The isolation, from critical chromosomal regions, of cosmic probes, which produce strong and discrete signals will allow more reliable and rapid detection of abnormalities than is possible through conventional banding. The introduction of the primed in-situ labelling (PRINS)[77] which utilises a specific unlabelled synthetic oligonucleotide primer hybridized to the denatured chromosomes or interphase nuclei, which is then elongated simultaneously introducing non-radioactively labelled nucleotides which are then detected directly or indirectly, is another alternative to the traditional FISH. Development of multicolour FISH will make reporting time in prenatal diagnosis reduced to a few hours and commercial availability of probes will make the technique one of the most widely used in laboratories all over the world.

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