CHAPTER III

FISH APPLICATIONS IN CYTOGENETICS

Introduction to Molecular Cytogenetics: FISH

Fluorescence *in situ* hybridization (FISH) has become an important adjunct to routine karyotyping and has greatly expanded the power of cytogenetic analysis. A major limitation of karyotyping is that it is applicable only to cells that are dividing or can be induced to divide in vitro. This problem can be overcome with DNA probes that recognize chromosome-specific sequences. Such probes are labeled with fluorescent dyes and applied to interphase nuclei. The probe binds to its complementary sequence on the chromosome and thus labels the specific chromosome, which can be visualized under a fluorescent microscope. Thus, FISH can be used to enumerate chromosomes in interphase nuclei.

The application of FISH is not limited to interphase nuclei, however. By using DNA probes that are specific for defined regions of the chromosomes, FISH can be used to demonstrate subtle microdeletions, complex translocations, and telomere alterations that are not readily detectable by routine karyotyping.

In addition to its diagnostic utility, FISH can also be used as a tool to physically map newly isolated genes of clinical interest. Novel DNA sequences are labeled with a fluorescent dye and then applied to a metaphase spread. The DNA binds to its complementary sequence and thus pinpoints the localization of the gene to a specific site.

Chromosome painting is an extension of FISH, whereby whole chromosomes can be labeled with a series of fluorescent DNA probes that bind to multiple sites along a particular chromosome. The number of

chromosomes that can be detected simultaneously by chromosome painting is limited by the availability of fluorescent dyes that excite different wavelengths of visible light. Thus, chromosome painting has limited ability to visualize all 46 human chromosomes simultaneously. This hurdle has been overcome by the introduction of multicolor FISH (mFISH). By using a combination of five fluorochromes and appropriate computer-generated signals, the entire human genome can be visualized.

Another recent FISH application directly compares the DNA content of differentially labeled normal and tumor cell populations by their co-hybridization to normal metaphase chromosome spreads (comparative genomic hybridization or CGH) or to a series of genomic DNA clones aligned on glass slides (array comparative genomic hybridization). In this manner, tumor-specific alterations in gene copy number can be ascertained.

FISH is transforming the way we study chromosomal changes in humans. It has improved the detection of microdeletion syndromes. *De novo* derivative chromosomes in patients with chromosome imbalances can now be accurately characterized: the clinician now knows the identity of the partial monosomy and trisomy in these patients, and the molecular geneticist has accurate information from which to find genes relevant to the phenotype. The chromosomal origin of supernumerary markers can be identified and we now have a real potential of predicting phenotype for marker carriers, benign or adverse.

With molecular cytogenetic techniques, we can now detect some chromosomal abnormalities in nondividing cells with interphase nuclei. Standard cytogenetics requires actively dividing cells with metaphase nuclei. Some constitutional chromosomal abnormalities and phenomena such as

mosaicism and chimerism can be addressed without growing cells in culture or in cell types that do not adapt well to tissue culture.

Potentially, chromosomal aneuploidy studies (i.e. studies looking for abnormalities in chromosome number) can be done more quickly. Preserved, rather than fresh, tissue can be investigated for retrospective studies. We can ask questions of chromosome organization, its relationship to gene expression and tissue specificity in ways that were not possible a few years ago.

Molecular cytogenetic techniques focus on specific chromosomes, chromosome regions, and unique DNA sequences or genes. Standard cytogenetic studies, including high resolution analysis, allow us to survey the whole genome for abnormalities of chromosome number or structure. But as complementary studies or for certain targeted abnormalities, molecular cytogenetic techniques expand our capabilities for making more accurate and refined cytogenetic diagnoses, both for constitutional abnormalities and acquired chromosomal changes in cancer cells.

Basic Techniques

The most commonly used molecular cytogenetic technique is fluorescence *in situ* hybridization, or FISH. FISH is usually applied to standard cytogenetic preparations on microscope slides, but it can be used on slides of formalin-fixed tissue, blood or bone marrow smears, and directly fixed cells or other nuclear isolates. The basic principle of the method is that single-stranded DNA will bind or anneal to its complementary DNA sequence. Thus, a DNA probe for a specific chromosomal region will recognize and hybridize to its complementary sequence on a metaphase chromosome or within an interphase nucleus. Both have to be in single-strand conformation, therefore

the DNA probe and the target DNA must be denatured, usually by heating them in a formamide-containing solution.

The probe is hybridized to the target DNA under conditions that allow the DNA to reanneal in double-strand form. Added to the hybridization mixture is an excess of repetitive sequence DNA to block non-specific binding of the probe to the target. After hybridization is complete (often after 2-18 hr at 37 ^{o}C), the slides are washed in formamide-saline citrate solutions to remove excess or non-specifically bound probe. To detect the location of the probe on the target DNA, the probe DNA can be directly labeled with a fluorescent tag. It can also be chemically modified by the addition of hapten molecules (biotin or digoxigenin) that can then be indirectly fluorescently labeled with immunocytochemical techniques. The target DNA is counterstained with another fluorochrome of a complementary color.

The probe DNA can be observed on its target by using a fluorescent microscope with filters specific for the fluorochrome label and the counterstain. Special filters have been developed to allow simultaneous visualization of several fluorochromes. Digital cameras designed to detect low light level emissions and computer imaging program are used to increase the sensitivity of probe detection. Because fluorescent dyes are subject to photobleaching (fading), the preparations are not permanent and must be stored away from light. Use of an antifade solution (phenylenediamine) has improved the capacity to observe and document fluorescently labeled samples.

Three different types of probes are typically used in clinical FISH studies:

1) **Alphoid or centromeric repeat probes** are generated from repetitive sequences found in the middle of each chromosome. Researchers use these

probes to determine whether an individual has the correct number of chromosomes. These probes can also be used in combination with "locus specific probes" to determine whether an individual is missing genetic material from a particular chromosome.

Probes that bind to specific chromosome structures typically recognize repetitive DNA sequences, such as within centromeres (alpha satellite DNA) or telomeric sequences. Within the repeat sequence are nucleotide patterns that are unique for specific chromosomes. There are now available centromeric probes that can identify most of the individual chromosome homologues. Similarly, there are probes that specifically recognize the telomeres (chromosome ends) of the long and short arms of many of the homologues, and more are rapidly being developed. Most alpha satellite centromeric probes give a large, bright signal and are useful for both chromosome identification in metaphase preparations and chromosome enumeration in interphase nuclei. Chromosome-specific telomere probes can be used for the above, for detection of cryptic translocations, and to define interstitial and terminal deletions.

2) **Locus specific probes** bind to a particular region of a chromosome. This type of probe is useful when scientists have isolated a small portion of a gene and want to determine on which chromosome the gene is located.

Unique sequence probes hybridize to single copy DNA sequences in a specific chromosomal region or gene. In clinical cytogenetics these probes are usually referred to as cosmids, named for their cloning vector. These are the probes used to identify the chromosomal critical region or gene associated with microdeletion syndromes. On metaphase chromosomes, they hybridize to each chromatid, usually giving two small, discrete signals per chromosome.

3) Whole chromosome probes are actually collections of smaller probes, each of which binds to a different sequence along the length of a given chromosome. Using multiple probes labeled with a mixture of different fluorescent dyes, scientists are able to label each chromosome in its own unique color. The resulting full-color map of the chromosome is known as a spectral karyotype. Whole chromosome probes are particularly useful for examining chromosomal abnormalities, for example, when a piece of one chromosome is attached to the end of another chromosome.

Whole chromosome paints are cocktails of unique sequence probes that recognize the unique sequences spanning the length of a particular chromosome. At metaphase, both chromosome homologues are "painted" or fluoresce brightly. Among other applications, paint probes are used to define the chromosomal origin of derivative segments on translocation chromosomes or supernumerary markers.

Because of the large number of cytogenetic analyses and the complexity of chromosomal rearrangements, which can be encountered, it is not surprising that fluorescence *in situ* hybridization (FISH) has been increasingly used to study chromosomes. In general, FISH has several advantages: Firstly, it offers the prospect to automate steps involved in karyotype analysis. Secondly, for a number of structural rearrangements the resolution of FISH is superior to that of classical banding analysis. Thirdly, FISH can be done independently of the cell cycle as signals can be visualized in intact interphase nuclei.

FISH is ideally suited for the simultaneous detection of multiple hybridization probes because of the availability of spectrally distinct fluorochromes. In addition, FISH allows the accurate quantitation of hybridization signals. These characteristics paved the way for multicolor-

applications, which evolved to the simultaneous hybridization of 24 or even more DNA-probes for the FISH-based karyotyping of chromosomes. For this, several techniques, including multicolor FISH (M-FISH), spectral karyotyping (SKY), combined binary ratio labeling (COBRA), or color-changing karyotyping have been developed. In addition, FISH-based banding technologies, such as cross-species color banding or high resolution multicolor-banding were developed for karyotyping and to facilitate the identification of intrachromosomal rearrangements. Thus, there is now an impressive arsenal of different multicolor-FISH technologies available.

mFISH Technology and Applications

1. mFISH Approaches Using Human Whole Chromosome Painting Probes

The applications of mFISH using whole chromosome libraries as probes cover the whole spectrum of human cytogenetics. In clinical genetics the technique has been used for the confirmation, refinement and/or characterization of translocations, search for cryptic rearrangements, and characterization of marker chromosomes.

In tumor cytogenetics karotypes and marker chromosomes of leukemias, lymphomas, solid tumors and cell lines have been studied.

2. Approaches to Distinguish Chromosomal Subregions in or by High Resolution Multicolor Banding (mBAND)

mFISH methods using human whole chromosomes painting probes reach their limits, when the exact breakpoint localization of translocations are required, or in case of intrachromosomal rearrangements such as interstitial deletions or inversions. Thus, different approaches have been developed to overcome those limitations. A high-resolution multicolor-banding (mBAND) technique based on five different fluorochromes has been described on the example of chromosome #5 by Chudoba and co-workers in 1999, allowing the

differentiation of chromosome region specific areas at the band and sub-band level. This technique is based on changing fluorescence intensity ratios along the chromosomes, which are used to assign different pseudocolours to specific chromosomal regions.

3. mFISH Approaches using Locus-specific Probes

mFISH methods can also be performed using locus-specific single-copy (i.e. cosmids, P1-clones, BACs, YACs) and/or juxta-centromeric probes. Apart from the applications for special scientific approaches like the characterization of chromosomal subregions using mFISH on chromosome fibers (fiber-FISH) locus-specific probes are used in the special mFISH approaches listed in the following.

3.1. mFISH with Subtelomeric Probes

The extreme ends of all vertebrate chromosomes consist of noncoding tandemly repeated hexanucleotide units TTAGGG (5'→3' direction). As in up to 10% of patients with idiopathic mental retardation cryptic subtelomeric translocations or deletions can be detected, efforts have been made to develop a set of subtelomeric probes suitable for FISH. The integration of these probes in mFISH has been suggested, because subtelomeric sequences are often underrepresented in the whole chromosome painting probes.

3.2. mFISH using Locus-specific Probes for Special Applications

Similar to the problem addressed with the subtelomeric probe set for idiopathic mental retardation due to cryptic subtelomeric translocations or deletions, other mFISH probe sets using single-copy and/or centromeric probes have been developed for the diagnostic approaches. As many microdeletion and contiguous gene-deletion syndromes include mental retardation as a clinical feature, a "MultiFISH" assay has been proposed to simultaneously screen for Prader-Willi/Angelman (15q11-13), Williams-Beuren

(7q11.23), Smith-Magenis (17p11.2) and DiGeorge/velocardiofacial (22q11.2) syndromes.

The use of mFISH techniques in uncultured amnion cells for the rapid interphase analysis of the most frequently occurring trisomies (#13, #18, #21) and numerical gonosomal aberrations is nowadays a quite often applied approach in prenatal diagnostics. The available mFISH kits are either 2-3 color FISH-assays (AneuVysion, Vysis, USA; Q-BIOgene, Illkirch, France) or 5 color FISH-assays (MultiVysion PGT, Vysis, USA) including two locus-specific single copy probes on 13q14 and 21q22 and three juxta-centromeric probes. For preimplantation diagnostics a further specific mFISH assay (5 colors) is available including three locus-specific single copy probes on 13q14 and 21q22 and 22q11 and two juxta-centromeric probes on #16 and #18 (MultiVysion PB, Vysis, USA). Moreover, mFISH probe sets with up to 10 single-copy and juxta-centromeric probes for the gonosomes, #9, #13, #14, #15, #16, #18, #21 and #22 have been created for preimplantation diagnostics, with the goal to detect up to 70% of the most frequent numerical chromosome aberrations responsible for spontaneous abortions.

3.3. mFISH using Alphoid Probes (cenM-FISH)

The first mFISH approach using three different alphoid probes at the same time has been described in 1996. Afterwards, many studies reporting on the aneuploidy rate in human sperm cells e.g. after exposure to mutagens, have been published. Another frequently studied field using three juxta-centromeric probes simultaneously is prenatal and preimplantation diagnostics using alpha satellite probes for the chromosomes X, Y and #18 as mentioned above.

Centromere specific multicolor FISH (cenM-FISH or CM-FISH) is a recently developed multicolor FISH technique which allows the simultaneous characterization of all human centromeres using labeled juxta-centromeric

satellite DNA as probes. CenM-FISH distinguishes all centromeric regions apart from the evolutionary highly conserved ones on chromosomes 13 and 21 in one single step by individual pseudo-coloring. The usefulness of the cenM-FISH technique for the characterization of small supernumerary marker chromosomes with no - or nearly no - euchromatin and restricted amounts of available sample material has been demonstrated in prenatal, postnatal and tumor cytogenetic cases. Moreover, rarely described markers with involvement of heterochromatic material inserted into homogeneously staining regions could be identified and characterized using the cenM-FISH technique.

Advantages and Limitations of the Mentioned mFISH Techniques

All mentioned mFISH techniques have their specific advantages and limitations. For example it has been stated, that mFISH approaches using human whole chromosome painting probes, like mFISH, SKY or COBRA are helpful in many cases of marker chromosome characterization. But one of the limitations to be mentioned is the need for highly sophisticated image acquisition and the image analyzing hard- and software are still not present in every laboratory.

Irrespective of applying SKY, COBRA or mFISH with human whole chromosome paintings as probes, these techniques are either not able to resolve exact breakpoint localization of translocations or are inadequate to detect intrachromosomal rearrangements such as interstitial deletions or inversions. Due to that, efforts have been made either to combine mFISH/SKY with conventional banding methods, to integrate banding into mFISH or to develop completely new mFISH based banding methods. Using the mBAND technique the breakpoints can be determined more precisely. For mBAND it has been demonstrated, that a resolution of 550 bands per haploid karyotype

is standard, irrespective of the condensation grade of the hybridized chromosomes.

Single probes, like cosmids, BACs, YACs and P1 clones can either be used in combination with other mFISH approaches – like e.g. with mFISH or with mBAND to confirm breakpoint or deletion mapping – or for specific clinical and tumor cytogenetic questions.

Probe sets with locus-specific probes for the subtelomeric and the juxta-centromeric region (cenM-FISH and CM-FISH) have been developed for complementation to all the other mFISH probes for "covering the whole human karyotype". mFISH, SKY and the mBAND techniques do neither cover juxta-centromeric heterochromatic material of human chromosomes nor are suited to detect subtle telomeric aberrations. The centromeres are not visible as chromosome *in situ* suppression of labeled repetitive sequences is done and repetitive sequences present in centromeric regions of human chromosomes also become suppressed by this technique. The subtelomeric regions are not covered sufficiently due to the complexity of the used probes.

Clinical Applications of FISH Technology in Cytogenetics

1) Microdeletion syndromes
2) Characterization of chromosome structural abnormalities
3) Aneuploidy detection
4) FISH and cancer cytogenetics
5) Gene mapping
6) Comparative genomic hybridization

Microdeletion syndromes

Syndromes such as Prader-Willi syndrome or Smith-Magenis syndrome were first characterized as chromosome deletion syndromes by high resolution cytogenetic analysis of prometaphase chromosomes. These deletions are small and often difficult to detect, or in the case of Williams syndrome, undetectable on prometaphase chromosomes. The term microdeletion syndromes is commonly applied to these.

Cosmid probes have been developed that recognize unique DNA sequences within the critical region of chromosomal deletion for several of the microdeletion syndromes. These probes, and their commercial availability, have made the laboratory diagnosis of these syndromes more accurate. In some cases, such as Velocardiofacial/DiGeorge syndrome, the prevalence of the deletion 22q11.2 both *de novo* and familial, in the population had not been appreciated.

In many labs, FISH is now the test of choice to document these deletions because it is more accurate, and arguably faster to perform. The tests are performed on metaphase chromosomes. Usually the microdeletion cosmid (locus-specific) and a second cosmid (chromosome-specific) are hybridized together to insure identification of the chromosome and to provide an internal control.

Characterization of chromosome structural abnormalities

Combinations of FISH probes and standard cytogenetics can characterize structural rearrangements and marker chromosomes. Take the example of a patient identified in the newborn period who has multiple congenital anomalies consistent with possible trisomy of the short (p) arm of #9. Standard cytogenetic studies demonstrate a structural abnormality of 9q: it

is twice the length of the normal homologue. mFISH probe hybridizes to the normal #9, and to most of the abnormal #9. This helps to confirm a duplication of chromosome 9 material which the G-banding pattern was difficult to interpret. Subsequently, the mBAND was performed to characterize the band of the duplicated segment of chromosome 9 and the results demonstrated that the duplicated segment came from 9p13→9pter. Subtelomeric FISH confirmed the subtelomeric deletion at insertion breakpoint on long arm of derivative chromosome 9. (see Appendix A)

This kind of diagnosis would not have been confirmed prior to the advent of clinical molecular cytogenetics. FISH is also teaching us to expect the unexpected: abnormalities are often more complex than they first appear.

Aneuploidy detection

The autosomal trisomies 21, 13, and 18, together with sex chromosomal aneuploidies, are responsible for well over 90% of the chromosomal disorders found in human conceptuses. They are easily diagnosed by standard metaphase cytogenetic studies of chorionic villus samples (CVS), amniotic fluid cells, and peripheral or cord blood lymphocytes, or other tissue that can be grown in tissue culture.

Chromosome-specific cosmids or alpha satellite probes can be used in FISH studies to document chromosome gain or loss in cells that are not dividing. Interphase cytogenetics using FISH is a rapidly growing field. One can enumerate the number of fluorescent signals present in interphase nuclei, for instance using an alpha satellite probe for the centromere of chromosome 18, or a unique sequence cosmid for a locus on chromosome 21.

Some laboratories offer interphase FISH aneuploidy studies of prenatal samples and for newborns suspected of having an autosomal trisomy.

Currently, these studies are used for screening, not as a stand-alone diagnostic test. It is the opinion of most geneticists that interphase FISH studies must be performed in conjunction with standard cytogenetic testing for clinical diagnosis. The results must be interpreted with caution, and appropriate controls must be developed.

Interphase FISH can be applied to uncultured cells and fixed tissue. An interesting use is the detection of tissue limited mosaicism of an aneuploid cell line, for instance in placental tissue or in buccal mucosa. Again, the technology is driving a new appreciation of the extent of chromosomal mosaicism to be found in humans.

FISH and cancer cytogenetics

The study of the acquired chromosomal abnormalities in cancer cells continues to expand. Cancer cells are often difficult to grow in culture and FISH techniques can augment standard cytogenetic testing. Numeric changes such as trisomy 8 in myeloid disorders can be detected by interphase FISH. Certain translocations, such as the *bcr/abl* rearrangement in chronic myelogenous leukemia and acute lymphoblastic leukemia can be detected by FISH on both metaphase preparations and interphase nuclei.

FISH studies are being used to look for early relapse and residual disease in nondividing cells. In allogeneic bone marrow transplant patients who received opposite sex donor cells, the success of engraftment can be monitored by FISH chimerism studies. Differentially labeled X- and Y-specific probes can be used to detect the proportions of XX to XY cells in bone marrow or peripheral blood nuclei in a dual color FISH procedure. By combining immunocytochemical detection of cancer cells and FISH techniques, chromosomal abnormalities and cell type can be simultaneously studied.

Gene mapping

FISH is used to map genes to specific chromosomes and chromosomal regions. The order of genes or gene sequences within a chromosome can be established by labeling the probes with different fluorochromes and detecting their hybridization color pattern. In a new technique called fiber FISH, chromosome-specific chromatin fibers can be spread on a slide and then hybridized with locus-specific probes to allow fine resolution mapping of DNA sequences.

Comparative genomic hybridization

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique that combines FISH and standard cytogenetics to look for genome-wide DNA amplification and deletions in tumor cells or cells of a patient with a possible chromosomal disorder. Its value is that unlike standard cytogenetics, cell culture is not necessary. Like standard cytogenetics, chromosome gains and losses, and amplification or deletion of chromosome regions can be detected without having to selectively FISH for a specific chromosome or sequence. Its use is currently in the research lab. Issues of sensitivity and reliability are being addressed. However, as with other molecular cytogenetic techniques, one year's experiment is next year's clinical test.