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Review Article

Fluorescence In Situ Hybridization: A Brief Review

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Fluorescence in situ hybridization (FISH) is used for many purposes, including analysis of chromosomal damage, gene mapping, clinical diagnostics, molecular toxicology and cross-species chromosome homology. FISH allows an investigator to identify the presence and location of a region of cellular DNA or RNA within morphologically preserved chromosome preparations, fixed cells or tissue sections. This report describes in situ hybridization, and discusses the past, present and future applications of this method for genetic analysis and molecular toxicology. © 1996 Wiley-Liss, Inc.

Key words: fluorescence in situ hybridization, mapping, chromosome painting, applications, methods, review

HISTORICAL PERSPECTIVE AND OVERVIEW

In situ hybridization (ISH) was first described by Pardue and Gall [1969a] and independently by John et al. [1969]. Using Drosophila salivary gland chromosomes as target, radiolabeled 5S, 18S and 28S rRNAs were hybridized and detected using photographic emulsion and mapped to chromosome 2. Later, Pardue and Gall [1969b] hybridized tritium-labeled mouse satellite DNA onto mouse metaphase chromosomes and showed that the majority of repetitive sequences in mouse exist at the pericentromeric regions. Hybridization technology at this time was very limited as molecular cloning techniques did not yet exist.

By the mid to late 1970s DNA [Southern, 1975] and RNA [Alwine et al., 1977] blotting hybridization methods were developed to detect the presence of a segment of DNA or RNA. Both techniques involve transferring electrophoretically separated DNA or RNA extracts onto a nitrocellulose filter or nylon membrane followed by hybridization of a labeled specific probe.

Originally, autoradiography with tritium radiolabeled probe was used to detect hybridization. Radiolabels now come in a variety of isotopes and elements depending on the need of the investigator (Table 1). Since their development [Landegent et al., 1984; Pinkel et al., 1986, 1988] fluorescent labels have steadily replaced radioactivity. Fluorescence has several distinct advantages over isotopic labeling, including speed of microscopic analysis, sensitivity, stability, safety, diminished hybridization time, multiple labels (multiple colors) and ease of use. Fluorescence-based hybridization has become so common that various commercial kits are available [Walsh et al., 1992].

By providing an alternative to radioactivity, FISH has proved to be an excellent tool for genetic analysis. Most strategies involve labeling cloned DNA using various means, including nick translation, the polymerase chain reaction (PCR) and random primer extension. The labeled DNA is then hybridized onto metaphase and interphase cells. Since target regions are extremely small relative to the chromosomal content within a nucleus, indirect immunological methods are typically employed to amplify the signal for detection and visualization. Haptens (e.g., biotin and digoxigenin), fluorochromes conjugated to avidin, and antibodies are frequently used in immunochemical fashion to detect hybridization.

By the 1980s, cloning emerged as a feasible way to derive unique probes. Since then, advances and innovations in molecular biology and instrumentation have increased the breadth and versatility of the hybridization methods. PCR has significantly reduced the time and labor required to produce large quantities of labeled probe. Microscopes which now have sophisticated optical designs, and multiband pass filters are commonplace. Cooled charge coupled device (CCD) cameras and computer enhanced imaging are now used to detect hybridiza-

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TABLE I. Comparisons of Labels*

Label	Sensitivity	Resolution	Speed (days)
^{32}P	med	low	7
^{35}S	high	med	10
^{3}H	high	high	14
Biotin	med	high	0.16
Digoxigenin	high	high	0.16

*Data obtained from Polak and McGee [1991].

tion signals that might otherwise be unnoticed. Flow cytometry, originally performed for analysis of cells, is now used to purify chromosomes to develop painting probes.

These advances have contributed to the widespread use of FISH for analysis of somatic and germ cell aneuploidy, gene and cosmid mapping and ordering, identifying genomic imbalances and screening putative transgenic animals. The sensitivity, power and speed of analysis assure the use of FISH for these and other applications for the foreseeable future.

HYBRIDIZATION REACTION: KINETICS AND COMPONENTS

Hybridization is a dynamic reaction where denatured target sequences (in solution, immobilized on filters or membranes, or within fixed tissue) and complementary single stranded DNA or RNA probes anneal forming stable double stranded hybrid molecules. During hybridization, stable and fleeting interactions between target and probe occur. Four types of duplexes are formed including target:target, probe:probe, stable (specific) hybrids, and unstable (nonspecific) hybrids. The first three types are more stable and their duplexes can withstand more stringent conditions during hybridization and subsequent washes due to a high degree of complementary base pairing. Probe:probe or target:target duplexes are not detectable or desirable because the complex is either not bound to the slide and is removed during the wash steps, or is not labeled and cannot be seen. Nonspecific interactions are usually eliminated during stringent wash steps.

The kinetic molecular theory is useful for predicting the parameters of the hybridization process. The concentration of probe and target, temperature and salt content, as well as pH, significantly determine the rate of annealing. Most DNA samples exist at ground state conformation in continuous double helices with minor looping, breaks and imperfections (noncomplementary base pairing). Duplex DNA is asymmetrical in sequence, excluding repetitive elements, and remains at ground state in cellular conditions. However, under alkaline (pH > 10) or low salt concentrations (0.01 M NaCl) in the presence of heat and/or formamide, DNA may be denatured [Darnell et al., 1990]. Upon returning to cellular conditions, the denatured DNA will form complementary base pairs, returning to its native conformation. Hybridization reactions are usually performed at 37°C in the presence of 2 × SSC (saline sodium citrate), 50% formamide, blocking, carrier and probe DNA, and intermolecular crowders, at pH = 7.0. For a detailed method and recipes see Tucker et al. [1994]. Under constant concentrations of probe and target, there are two major (formamide and temperature) and several minor influences determining the rate and extent of hybridization.

Temperature and Salt Concentration

Hybridization between probe and target occurs just below their melting temperatures (T_m). The T_m of any given strand of duplex generally depends on three factors: base composition, length and salt concentration [Schildkraut and Lifson, 1965]. This relationship is expressed in an equation which predicts the T_m for any given strand of duplex DNA:

$$T_m\ (°C) = 16.6 \log M + 0.41\ (\%\ G + C) + [81.5 - (820/l) - 1.2\ (100 - h)]$$

where M is the monovalent cation concentration, % G + C is the base composition, l is the duplex length, and h is the percent interstrand homology [Lathe, 1985]. For probes with a G + C content near 50%, in the presence of 6 × SSC or below the above equation effectively reduces to

$$T_m\ (°C) = 102 - 820/l - 1.2\ (100 - h)$$

Due to the additivity of hydrogen bond energies across base pairs, the enthalpy required to break those bonds is directly related to length of the duplex. Because GC base pairs have three hydrogen bonds between bases compared to two for AT base pairs, the T_m increases as the molar GC content increases. For DNAs of heterogeneous sequence greater than 1 kb, the sequence effects are diminished. However, for short strands or repetitive elements the GC content will significantly determine the T_m.

Melting temperature of duplex DNA is also a function of salt concentration. SSC in the hybridization mixture stabilizes noncomplementary base pairing. The monovalent cation Na^+ interacts electrostatically with the negatively charged phosphate backbone of the nucleic acids. By neutralizing the charge of an oligonucleotide strand, the repulsion between target and probe is minimized, thus stabilizing a hybrid.

Formamide

Formamide is used during hybridization because it destabilizes hydrogen bonding across base pairs and lowers

the melting temperature (T_m) of target and probe. Within the range of experimental conditions, each percent formamide present lowers the T_m by about 0.7°C [Polak and McGee, 1991]. Formamide destabilizes AT-rich sequences disproportionately more than GC-rich hybrids. A disadvantage of formamide is that the hybridization reaction time is increased by approximately threefold [Sambrook et al., 1989].

Blocking DNA

Blocking DNA is often used to reduce nonspecific hybridization of the probe to nontarget DNA. It consists of repetitive sequences or whole-genomic extracts sized between 400 bp and 1 kb (usually 70 to 170 ng/mL hybridization volume). The concentration of blocking DNA relative to probe DNA during hybridization varies (3× to 100×). However, since the concentration of labeled probe for a given locus greatly exceeds that of the blocking DNA, the hybridization signal is not diminished significantly. The hybridization rate actually may be increased by using blocking DNA as it competitively inhibits probe hybridization to nontarget sites of the cells. As an adjunct to blocking repetitive elements in target cells with DNA, the target and probe may be incubated separately for 10–30 min at 37°C following denaturation. This permits the repetitive elements of the DNA to anneal, and decreases undesirable hybridization signals.

Carrier DNA

DNA from distantly related species (salmon or herring sperm) is often used as carrier DNA during hybridization in order to reduce background. Carrier DNA associates with nonbiological sites (e.g., microscope slide, filter or membrane) in the absence of target [Sambrook et al., 1989]. Carrier DNA is sized to approximately 400 bp and usually 70 to 170 ng/mL (up to 30-fold the concentration of probe) is used during hybridization.

Probe

The optimal probe length and concentration should be determined experimentally. Typical probe length is between 250 bp and 1 kb, depending on the application. We have found that 200 to 800 bp is the optimal length for chromosome painting [Breneman et al., 1994] and mapping. Probes exceeding 1 kb usually result in increased nonspecific background, thus decreasing the signal to noise ratio. Probes less than 200 bp may have insufficient hydrogen bonding to the target and require lower stringency during hybridization and washes for adequate signal detection.

Probe concentration often affects the signal to noise ratio. If the probe concentration is too high, non-specific background hybridization is increased and decreased signal to noise is observed. Alternatively, if the probe concentration is too low, hybridization is insufficient for detection. Usually 1.5 to 50 ng/mL of probe in the hybridization mixture is used during hybridization. For repetitive targets (e.g., pericentromeric sites) or whole chromosome painting, less probe (1.5 to 7 ng/mL) is required for adequate signal. However, when using multiple probes simultaneously, or mapping a small target (40 kb) the concentration of probe often exceeds 40 ng/mL.

Intermolecular Crowders

Crowders (e.g., dextran sulfate, gelatin or bovine serum albumin) facilitate the hybridization process by decreasing the free space in the hybridization reaction. Dextran sulfate is commonly used because it is more hydrophilic than DNA or RNA, and is strongly hydrated in aqueous solutions. Thus, the nucleic acids have less access to water and hybridize at a faster rate.

pH

In normal cellular conditions (pH = 6 to 8) the rate of hybridization is independent of pH. Therefore, buffers containing 20 to 50 mM phosphate, pH = 6.5 to 7.5 are used frequently throughout the FISH process [Tucker et al., 1994a]. At higher and lower pH alkaline denaturation and hydrolysis of DNA occur, respectively.

Hybridization Time

For most applications, hybridization overnight (12 hr) is the minimum time necessary for observing adequate hybridization signal. Longer times (up to 3 days) may help to increase signal intensity; however, this may result in decreasing the signal to noise ratio. For repetitive elements, 2 to 5 hr may be all that is necessary. The optimal hybridization time should be determined experimentally.

PRODUCTION OF DNA FOR USE AS PROBE DURING FISH

Cloning

Much of the FISH analysis used today uses cloned DNA. Cosmids, plasmids, YACs and PACs may be used to produce suitable probe. Often, the insert together with vector may be labeled using nick translation, PCR or random-primer extension. Alternatively, the cloned insert may be removed with restriction enzymes prior to labeling. The disadvantages of a cloned probe are few; once the vector containing the insert is isolated, the resulting DNA is an excellent probe source. However, generating the clone, i.e., locating, isolating and cloning the DNA, is time consuming.

Polymerase Chain Reaction

PCR is an excellent tool for generating and mass producing DNA for use as probe during FISH [reviewed by Narayanan et al., 1992]. First described by Saiki et al. [1985] and Mullis et al. [1986], PCR was used to amplify preferentially a segment of DNA which was flanked by known sequences. Today, variations of PCR including degenerate oligonucleotide primed PCR (DOP-PCR), linker-adapter, inter-alu, and primed in situ labeling (PRINS) are used routinely.

DOP-PCR

In 1990 Compton first described the use of DOP-PCR [Compton, 1990]. Later Telenius et al. [1992a,b] broadened the use of DOP-PCR for application in FISH. The degenerate primer used during PCR on flow sorted chromosomes produces a population of randomly amplified regions generally representative of the entire chromosome [Carter, 1994]. DOP-PCR uses two annealing temperatures (Ta) and multiple reactions, termed generations. The first PCR reaction consists of 5 to 15 cycles and uses a low stringency Ta (approximately 30°C) allowing the degenerate primer to anneal nonspecifically to many regions along template DNA. These products continue to be amplified during subsequent cycles in which more stringent annealing temperatures (e.g., 62°C) are used. The first generation products serve as template for the subsequent reactions. By incorporating a substituted nucleotide with label, an entire chromosome painting probe may be derived and used during FISH. Because the products of one reaction are used as template in subsequent reactions (generations), significant quantities of probe may be derived from a limited amount of original template.

Linker-adapter PCR

The linker-adapter PCR method provides sequence-independent PCR to be used for producing and amplifying probe [Vooijs et al., 1993; Miyashita et al., 1994]. After generating known restriction sites in isolated DNA, complementary sticky-ended linker-adapters are ligated to the products and used as flanking primer recognition sites for subsequent amplification. This method requires more effort than DOP-PCR for producing whole chromosome paints. However, it eliminates the need for cloning and may yield a more complex probe than DOP-PCR.

Inter-alu PCR

By using naturally occurring interspersed repetitive elements (LINES, SINES) as primer recognition sites for PCR, whole chromosome painting probes may be generated. Lichter et al. [1990] and later Lui et al. [1993] described the use of the *alu* family (SINE) as a scheme for generating human whole chromosome painting probes from CHO-human hybrid cells. Hybrid cell lines, many of which are commercially available, reduce the need for flow sorting. Disadvantages of this resource include the lack of a complete set of monochromosomal hybrids for every animal, the instability of the hybrid cell lines, and, for many species, a lack of knowledge concerning the chromosomal distribution and sequence of repetitive DNA. Furthermore, it has been our experience that small amounts, or pieces, of undesired chromosomes are sometimes present in so-called "monochromosomal hybrids," resulting in hybridization to other chromosome regions.

In humans, there are approximately 500,000 *alu* sites per haploid genome. Many of the paints derived from inter-alu PCR result in hybridization patterns that are discontinuous and often appear banded, coinciding with G-band light (gene-rich) regions. While alu banding may be used for identifying chromosomes, many alu-derived paints are not well suited for applications requiring whole chromosome painting for aberration analysis, as detection of subtle exchanges is difficult due to the banding.

While PCR is often the method of choice for generating and labeling probe, some disadvantages exist. Sequence knowledge is necessary to generate a probe, unless the DNA of interest has been previously isolated (e.g., by sorting or microdissection). Some modified nucleotides are difficult to incorporate during PCR, possibly due to low polymerase fidelity in relation to steric hindrances that these modifications cause in DNA. Finally, producing long fragments is effective up to approximately 20 kb, but products larger than 30 kb are difficult to generate.

Primed In Situ Labeling

The PRINS method [reviewed by Hindkjaer et al., 1991] incorporates label by primer elongation directly on a microscope slide [Koch et al., 1989]. PRINS is not well suited for unique sequence detection and is generally limited to repetitive targets [Volpi and Baldini, 1993]. Furthermore, the hybridized probe often becomes diffuse during the denaturation step, diminishing resolution.

Microdissection

Microdissection has been used for generating region-specific probes and whole chromosome-specific painting probes [Guan et al., 1993]. The desired chromosome region(s) are physically scraped with a glass micropipette tip and amplified by PCR. Microdissection eliminates the need for chromosome sorting and cloning, and can yield good probe. The disadvantages of this method are the lack of resolution when scraping, the need for sophisticated

equipment, a knowledge of karyotypes and the intensive labor it requires.

Flow Cytometry

Flow cytometry has facilitated the production of probes for FISH by allowing investigators to isolate chromosome specific DNA. High resolution flow sorters use advanced laser and optics technology to separate one or more chromosome types from a pool of prepared chromosomes [Shapiro, 1993]. Sorting involves less manipulation than cloning, so the DNA is less subject to contamination while the yield is hundreds of times greater than with microdissection. The major disadvantages of sorting are the high cost and limited availability of the technology.

HYBRIDIZATION DETECTION AND VISUALIZATION

Indirect Labeling Methods

The most common method for detecting hybridized probe involves the use of DNA labels that are not by themselves fluorescent, such as biotin and digoxigenin [Pinkel et al., 1986; Tucker et al., 1994a]. These molecules are covalently bound to a nucleotide and can be incorporated by a variety of methods including random primer extension, PCR and nick translation. To detect bound probe, macromolecular reporter molecules (e.g., avidin, antibodies) that are conjugated with a fluorescent tag such as fluorescein, rhodamine or Texas red are then applied to the microscope slide. Biotin labeled probe is typically detected with fluorescein-isothiocyanate conjugated avidin (avidin-FITC), and can be amplified with successive layers of biotinylated anti-avidin antibody and avidin-FITC. Digoxigenin is amplified with anti-digoxigenin antibody and can be amplified with a secondary antibody that is specific for the first antibody. The primary advantage of using indirectly labeled probes is the ability to amplify the signal, although there are limits as to the number of layers of avidin or antibody that can be added before nonspecific annealing (background) overwhelms the signal of interest.

Digoxigenin typically produces lower levels of undesirable background fluorescence than biotin, probably because biotin is a naturally occurring vitamin and may exist in small amounts on slide preparations, while digoxigenin is not a constituent of animal cells. However, digoxigenin can be more difficult to incorporate during nick translation or PCR and for this reason is not used as often. However, incorporation problems can often be overcome by increasing the incubation time for nick translation. Similarly, for PCR, excellent incorporation levels can be obtained by prolonging the primer extension step and lowering the annealing and extension temperatures by a few degrees.

Direct Labeling Methods

Directly labeled probes (e.g., FITC, rhodamine and various cyano dyes) are those in which the fluorochrome is covalently attached to the DNA [Wiegant et al., 1993]. These probes are advantageous because they do not require the use of avidin or antibodies for their detection, thereby avoiding many of the posthybridization washing steps and saving significant amounts of time and reagents. Other advantages of directly labeled probe are greater resolution and increased signal to noise ratio. Directly labeled nucleotides are commercially available from a variety of sources and come in many colors. The primary disadvantage of direct labeling is the limited amount of fluorochrome that can be incorporated into the probe and the inability to use antibodies or avidin for signal amplification. Enzymatic incorporation of fluorochrome-labeled nucleotides requires conditions that are similar to those for incorporation of digoxigenin-conjugated nucleotides.

Counterstains and Dyes

Aromatic diamidines, for example 4′,6-diamidino-2-phenylindole (DAPI), associate with DNA with great affinity [Zimmer and Wahnert, 1986]. These dyes are useful for identifying and observing chromosomal material and are a common adjunct to FISH [Schweizer, 1981; Tucker et al., 1988]. Dyes and fluorochromes with differing excitation and emission maxima may be observed simultaneously by using multiband pass filters, which are being used with increasing frequency.

DAPI binds strongly to the minor groove in DNA sequences containing three to four adjacent AT base pairs, allowing chromosomal identification by banding [Wilson et al., 1990]. However, as the ratio of DAPI:DNA increases, DAPI binds increasingly at GC-containing sites and banding is diminished. Propidium iodide (PI), which is similar in structure to DAPI, is a nonspecific duplex DNA and RNA binding dye. PI is also commonly used as a counterstain for fluorescein because both molecules are excited at the same wavelength (488 nm). However, they emit at different wavelengths, with PI and fluorescein appearing red and yellow, respectively.

Antifade Agents

Most fluorochromes will fade rapidly upon ultraviolet excitation. To alleviate this problem, chemicals that prolong the intensity of the photon emissions have been routinely used for many years [Johnson and Nogueiro Araujo, 1981], and are now commercially available (Vectashield, Vector Labs).

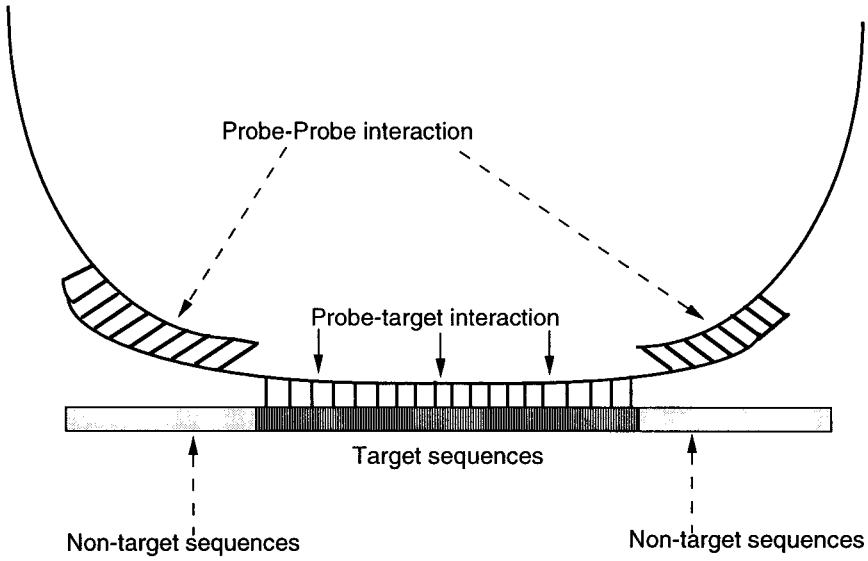

Fig. 1. Probe bridges.

Bridging

Bridges are probe-probe interactions that are anchored by probe-target hybridization, as shown in Figure 1. Probe bridges may occur during hybridization, increasing the sensitivity as well as the intensity of the hybridization signal. With bridging, the target site will have more labeled DNA hybridized to the region than would be expected according to the amount of sequence homology. This would have the effect of amplifying the hybridization signal. For this reason, cloned probe may be advantageous for applications such as cDNA mapping, where the target is small. Bridging is expected to increase the likelihood of hybridization detection, and deserves thought when designing a probe.

FISH Detection Limits

The power of the FISH method lies in the ability to visualize chromosomes or chromosomal regions with sensitivity and ease. Visualization of whole chromosomes, centromeric repeat sequences, and unique sequences as short as 40 kb are now routine. However, unique sequences shorter than a few dozen kb cannot always be detected reliably. Computerized image processing is often helpful, but not all laboratories have access to this expensive technology. The lower limits of resolution have yet to be clearly defined, but will almost certainly improve if brighter fluorochromes and improved hybridization methods become available.

APPLICATIONS OF FISH

This section describes briefly a few of the many applications of FISH. No attempt was made to cite every article on each subject. Instead, the intent is to provide a broad overview, and only a few seminal or representative references are provided.

Toxicology/Biodosimetry

Cytogenetic evaluations have been employed as a part of toxicological evaluations for many years [Preston et al., 1981; Tucker et al., 1993]. The advent of molecular cytogenetics has added the possibility of a new dimension to toxicity studies. Historically, toxicology studies which included cytogenetic endpoints were usually limited to single, high-dose acute exposures, and chromosome aberration analyses were conducted within a day or so. This was because the cytogenetic methods available at the time were only able to detect unstable (asymmetrical) chromosome rearrangements (dicentrics, rings, deletions, and fragments). With molecular cytogenetic methods, particularly whole-chromosome painting, stable (symmetrical) aberrations (translocations and insertions) can be quantified with ease and precision. As a result, chronic, low-dose cytogenetic evaluations are now feasible [MacGregor et al., 1995a,b] for these end points.

The field of radiation biology was quick to realize the value of FISH, especially when performed with whole chromosome paints. The relationship between chromosome aberrations and many types of ionizing radiation was well understood long before FISH. However, a major impediment to the study of radiation exposure in humans was the inability of conventional cytogenetic methods to provide quantitative exposure evaluations in a rapid and cost-effective manner. The exception, of course, was when a blood sample could be obtained within a few weeks of exposure (before dicentrics begin to decline),

but for many radiation accidents this has not been possible. Similarly, the ability to provide dosimetry under conditions of chronic exposure was limited. The ability of chromosome painting to quantify stable chromosome exchanges has enabled radiation biologists to perform meaningful dosimetry years after exposure.

However, for painting to be accepted as a valid method, it first had to be shown that it yielded measures of chromosome damage that were identical with established methods. Work by our laboratory and by others has shown that painting yields estimates of chromosome damage that are in very good agreement with conventional cytogenetic methods [Lucas et al., 1989; Natarajan et al., 1992; Schmid et al., 1992; Bauchinger et al., 1993; Tucker et al., 1993], at least at low doses of radiation. However, at high doses, the agreement is not as good [e.g., Tucker et al., 1995a], primarily due to a large number of cells that contain complex rearrangements involving multiple chromosomes [e.g., Griffin et al., 1995; Simpson and Savage, 1995]. Whereas such complex exchanges are easily identified using whole chromosome painting, they are not generally detected by more traditional methods. Recently a new nomenclature system was developed called Protocol for Aberration Identification and Nomenclature Terminology (PAINT) [Tucker et al., 1995b]. With PAINT it is now possible to classify every type of rearranged chromosome, regardless of the level of complexity.

Rodents as Model Systems

Currently many toxicology experiments utilize rodents as model systems to predict the effects of chemicals and radiation on humans. Whole chromosome painting probes for mice [Breneman et al., 1993, 1995; Boei et al., 1994; Rabbitts et al., 1995] and rats [Hoebee et al., 1994] have recently been developed, and their application to toxicological assays is expected to grow substantially. The advantages and disadvantages of whole chromosome paints for rodents are much the same as for the human probes. Chromosome region-specific rodent probes are also beginning to see utility, as seen for the human probes, and are particularly useful for detecting and quantifying numerical alterations [Wyrobek et al., 1995].

Germ Cell Analyses

FISH is being applied to study chromosomal alterations in sperm. Changes in chromosome number (aneuploidy) can now be elucidated clearly and unambiguously [Bischoff et al., 1994; MacGregor et al., 1995b; Wyrobek et al., 1994, 1995]. Thousands of sperm can be analyzed in a short time, yielding precise estimates of the frequencies of abnormalities. This work is particularly relevant because aneuploidy is a major cause of birth defects in humans.

Gene Mapping

FISH is an efficient and common method for mapping cloned genes. Used alone or in combination with other probes, physical mapping is relatively easy, accurate and reliable. Numerous genes and anonymous sequences have been mapped by FISH, and their relative order along a chromosome has been determined by simultaneous hybridization of different clones labeled in multiple colors. FISH has provided a valuable independent confirmation of the order of cloned probes determined by computational methods [Brandriff et al., 1994; Gordon et al., 1995].

Transgenic Screening

In some circumstances, FISH is a less labor-intensive method for confirming the presence of transgenic DNA than conventional methods such as Southern blotting. The method has been shown to work on peripheral blood smears from mice where the transgenic DNA exists in multiple copy numbers [Dinchuk et al., 1994; Swiger et al., 1995]. The primary advantage is the elimination of the need for cell culture, nuclei preparation, and DNA extraction and blotting. FISH-based assays appear to be more accurate than conventional screening techniques such as dot blots, and are easier to perform. Another major advantage of FISH is that it permits the determination of the zygosity status of the transgene-positive animals, something that eludes present PCR techniques.

Clinical Cytogenetics

FISH has been proven to have significant advantages in clinical cytogenetics. The ability to characterize aneuploidy, as well as complex or unusual structural rearrangements and marker chromosomes in children with birth defects and their families, represents an important advance in the clinical setting. Tumor diagnostics has also improved, for much the same reason. Many chromosome rearrangements are now known to be associated with specific tumors [Rabbitts, 1994]. The ability to detect structural rearrangements that are related to tumor progression should improve decision-making concerning treatment regimens.

Comparative Genomic Hybridization

Comparative genomic hybridization (CGH) is used to identify genomic differences between cell lines [du Manoir et al., 1993; Cher et al., 1994; Kallioniemi et al., 1994]. The method involves labeling the DNA of a tumor or other potentially abnormal cell type with one label and color (e.g., fluorescein, green) and the DNA of normal cells with another label (e.g., rhodamine, red). Equimolar

amounts of the two labeled DNAs are then hybridized simultaneously to normal cells. Differences in the amount of chromosomal material between the cell types are seen as alterations in the color of the hybridization along the length of each chromosome. In the example given here, regions of the tumor that are diploid will appear yellow because of the 1:1 hybridization ratio of red and green. Regions that are hyperdiploid (e.g., duplicated or amplified) will appear more green than yellow, while hypodiploid (i.e., deletions) regions will appear more red. CGH eliminates the need for cell culture, and searches entire genomes for hyper- and hypodiploidy in a single hybridization, with a resolution typically at the level of a chromosome band. One disadvantage of CGH is that the results for each chromosome band represent an average of the ploidy for the whole tumor, i.e. mosaicism cannot be detected with any degree of confidence. The second disadvantage is that CGH is not sensitive to detecting structural chromosome rearrangements.

Zoo-FISH

Zoo-FISH refers to cross-species in situ hybridization. The most common purpose is to identify regions of chromosome homology between species. Whole painting probes of one species are used as probe on prepared chromosomes of another species [e.g., Scherthan et al., 1994]. Under appropriate conditions (e.g., high probe concentrations, long hybridization times and non-stringent washing), weak to moderate cross-species hybridization signals can be detected. Zoo-FISH is more precise than chromosome banding for detecting regions of homology, and should provide much useful information concerning relationships between species.

Acetylation Banding of Chromosomes

Acetylation of duplex DNA has long been related to gene activity. Most of these reports use antibodies specific for the various subtypes of acetylated histones in order to infer global transcription [Jeppesen et al., 1992; Turner et al., 1992]. Recently, a method using histone-associated DNA during FISH has been reported [Breneman et al., 1996]. With this method, unique discreet banding patterns from DNA associated with high-, medium- and low-acetylated histones are observed.

CONSIDERATIONS FOR THE FUTURE

The sensitivity, specificity and speed of analysis of FISH has made it the method of choice to address a wide variety of problems. In spite of the obvious advantages of FISH compared to classical techniques, there are several enhancements that would further improve the sensitivity and specificity of the method. Specific areas where improvements would be particularly useful include the following. 1) An increase in the number of modified deoxynucleotides that are presently available, which would allow an increase in the number of colors that can be detected simultaneously. 2) An increase in the number of organic fluorochromes that emit distinctive colors in the visible range and can be covalently attached to avidin or antibodies, or directly to DNA. 3) Brighter fluorochromes, with greater resistance to fading upon exposure. This would allow detection of shorter sequences of hybridized DNA and could lead to highly detailed analyses of chromosomes, nuclei and other cellular components, as well as increasing the "shelf life" of analyzed microscope slides. 4) Development of efficient FISH techniques that do not depend heavily upon slide storage at −4°C in the presence of N_2 gas. Although these or other advances may be slow in coming, the power of FISH assures the continued use of this technology for many years to come.

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