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FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH) IN THE MOLECULAR CYTOGENETICS OF CANCER*

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In this review, we discuss the developments of fluorescence *in situ* hybridization (FISH) and place them in the context of their applications in cancer research. These methods are not only very useful for the causal analysis of the development and spread of certain tumors, they are also efficient tools for tumor diagnosis. Although a review of all of the literature in this field is not possible here, many of the major contributions are summarized along with recent work from our laboratory.

Our group contributes to the goal of functional identification of tumor growth antagonizing genes. FISH and molecular analyses have shown that the short arm of human chromosome 3 is frequently deleted in kidney, lung, breast, uterus, testis and ovary carcinomas. Deletion-mapping studies have outlined several separate deletion prone regions in different tumors, namely 3pter–p25, p22–p21.3, p21.1–p14 and p14–p12, which may contain putative tumor suppressor genes (TSGs). Candidate suppressor genes isolated from frequently deleted regions need to be assayed for possible tumor-antagonizing ability by functional tests. We have developed a functional test system, the microcell hybrid (MCH) based "elimination test" (Et). The Et is based on the introduction of a single human chromosome into tumor cells of human or murine origin, via microcell fusion. The MCHs were analyzed by FISH painting and PCR for the elimination or retention of specific human chromosome 3 (chr. 3) regions after one or several passages in severe combined immune-deficient (SCID) mice. We have defined a common eliminated region (CER) on chr. 3p2I.3. CER is approximately 1 megabase (Mb) in size. We have covered this region with PACs (bacteriophage PI based artificial chromosome) and used FISH mapping for localization and ordering PACs and cosmids on the chromosome 3 and high-resolution free chromatin/DNA fiber FISH to orient the PAC contig, to measure the lengths of PACs, and to establish their order.

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Activation of cellular oncogene by chromosomal translocation, which brings an oncogene under the influence of a highly active chromosome region, appears to play a pivotal role in the genesis of certain hematopoetic and lymphoid tumors. We have detected specific chromosomal translocations by FISH painting in mouse plasmacytoma (MPC), human Burkitt lymphoma (BL) and other B-cell derived tumors.

We have showed in a murine sarcoma derived line (SEWA) that FISH can also be used for detection of amplified oncogene (c-*myc*) and the linked locus (*pvt*-1).

We have also applied the FISH technique for visualization of integrated and episomal Epstein-Barr virus (EBV) genomes and EBV transcripts in EBV-carrying B-cell derived human cell lines.

Key words: fluorescence *in situ* hybridization, molecular cytogenetics, tumor biology

Introduction

The field of molecular cytogenetics has been revolutionized in recent years by advances in the technique of fluorescence *in situ* hybridization (FISH), which have provided powerful new tools for identification of chromosomes, detection of chromosomal abnormalities, localization of specific DNA sequences on the chromosome and genome-wide screening of alterations in copy number, structure, and expression of genes. The enormous potential of FISH derives from the unique ability of this approach to directly couple cytological and molecular information. FISH plays an increasingly important role in a variety of research areas, including cytogenetics, prenatal diagnosis, tumor biology, gene amplification and gene mapping. A major area in which it promises to have a large impact is in the investigation of genome organization, not only on chromosomes, but in the functional state within the interphase nucleus.

History of development

In situ *hybridization*

DNA *in situ* hybridization (ISH) involves the hybridization of nucleic acid probes to cytological preparations of chromosomes or chromatin. The earliest phase of *in situ* hybridization technology relied on autoradiographic detection of abundant sequences, such as localization of DNA in amplified polytene chromosomes or highly

reiterated sequences on metaphase chromosomes [1–3]. In 1981, two reports [4, 5] showed that it was possible to localize single sequences on metaphase chromosomes by autoradiography of ^{125}I- or ^{3}H-labeled probes using statistical analysis. Limitations are the relatively poor resolution, the fact that localization is not determined within a single cell but requires statistical analysis of many metaphases, and the time-consuming nature of autoradiography, which often requires several weeks.

Fluorescence in situ *hybridization (FISH)*

FISH was introduced in the late 1970s [6] as an alternative to the use of radioactive probes and signal detection by autoradiography. The probes were either conjugated directly with a fluorochrome or with a reporter molecule (e.g. biotin or digoxigenin) that after hybridization binds fluorescent affinity reagents. The signal was thereafter visualized in a fluorescence microscope [7–10].

Technique overview

The basic FISH technique involves preparation of cytological material, incorporation of labeled nucleotides into the DNA probes by standard labeling techniques, denaturation of the target chromosomes or nuclei and probe DNA (when the target cells or tissues are not denatured, only RNA will hybridize), followed by the hybridization of the single-stranded probe DNA to target DNA or RNA. Stable DNA-DNA or DNA-RNA hybrids will be viewed with epifluorescence optics [11].

Microscopy

Virtually any fluorescence microscope is suitable viewing the hybridization pattern of repetitive sequences, but high-quality microscopes are necessary to detect fluorescent signals from single-copy sequences. Both the sensitivity and flexibility of FISH techniques have been improved by the introduction of cooled charge-coupled device (CCD) cameras. These devices are extremely sensitive to photons and exhibit an almost perfect linear response to light. The exquisite sensitivity allows the detection of very small sequences. The devices also have a wide dynamic range, allowing multicolor imaging, with the number of fluorochromes detectable limited only by the number of fluorescent filter sets available on the microscope. Digital imaging microscopy also introduces the possibility of quantifying *in situ* hybridization signals. Nederlof et al. [10] optimized the instrumentation for quantitation of fluorescent signals, but were still hampered by significant variation in the size of signals. Labeling

with directly fluorochrome labeled dNTPs has been demonstrated recently to be applicable to the detection of single-copy sequences and subject to less background.

Probes

The type of material that can now be used readily as hybridization probe has expanded rapidly, ranging from small sequences of genomic DNA or cDNA through whole-chromosome "paints" to the whole genomes. Paints are now commercially available for all human chromosomes, as are probes for most centromeres, some telomeres and a range of specific loci. The principal advance in probe generation has been the ability to amplify selected DNA by PCR and to incorporate labels directly into the amplification reaction, with suitable primers selected according to the application.

Genomic

The sequences repeated most frequently in mammalian genomes show little evolutionary conservation. Because of their high frequency in probe and target, repetitive sequences reanneal more rapidly than more highly conserved unique sequences in the genome. Thus, when used as a probe, total nuclear (genomic) DNA can be a species-specific label. We have used rat genomic DNA that specifically labels rat chromosomal material in a mouse × rat somatic cell hybrid and also human genomic DNA for detection of human chromosomes in human mouse microcell hybrids. (See also in Ref. [8]).

Chromosome paints

Collections of DNA sequences derived from a single human chromosome type can highlight that chromosome in metaphase and interphase nuclei [12–14]. Collections can be derived from somatic cell hybrids carrying the desired chromosome as its only human material or from suspensions of chromosomes purified by flow sorting. The DNA is cloned, amplified by PCR between Alu or LINE sequence primers, or, in case of hybrid DNA, used directly for labeling. Prehybridization with unlabeled genomic DNA suppresses hybridization of repetitive elements in the collection that are common to many chromosome types.

Chromosome-specific repeats

Repetitive elements that are repeated 100–5000 times on specific chromosome types have been cloned for more than two-thirds of the human chromosome types [15].

When labeled and hybridized at sufficient stringency, these probes produce intense and compact zones of hybridization near centromeres or in heterochromatic regions of specific chromosome types and in compact domains in interphase chromatin [10].

Single-copy sequences

The efficiency of labeling unique sequences using large-insert probes (i.e. cosmids or yeast artificial chromosomes, YACs) is >90% [9, 16, 17] under suppression conditions. The efficiency of hybridization site detection decreases with decreasing probe size. However, despite low hybridization efficiencies (20–50%), plasmid probes containing as little as 2 kbp of target sequence have been localized using FISH [3–5].

Applications of FISH in cancer research

Chromosomal alteration and cancer

Genes involved in cancer development can be divided into three groups on the basis of their action;

1) Oncogenes, that are dominant in the sense that may directly induce neoplastic growth when activated.

2) Tumor suppressor genes, that when deleted or inactivated, promote tumor development.

3) DNA repair genes, that when mutated lead to genomic instability.

Alteration of the genetic material that led to changes in the function of oncogenes may be caused by chromosome alterations that are visible under the microscope. For example, the number of chromosome (numerical chromosome aberration) or their structure (structural chromosome aberration) may be specifically changed. Numerical changes and chromosomal rearrangements can be investigated with FISH in a highly specific manner.

Chromosomal alterations that are particularly important for cancer diagnosis in humans are translocations (broken chromosomes which subsequently fuse with material of different chromosomal origin) and deletions (lose of chromosomal regions). Other chromosomal changes as duplications (of chromosomal regions) and inversions (inverted reintegration of chromosome sections) also play a role. In certain types, specific oncogenes can be found in the breakpoint region of translocations typical for certain tumors. The effect of tumor suppressor genes can often be demonstrated by detecting deletions.

Whereas loss of heterozigosity (LOH) accompanied by mutations in the remaining allele, and homozygous deletions, are consistent with the notion of a tumor suppressor gene, the former genetic analysis needs to be complemented with functional tests. Suppression of tumorigenicity by the transfer of whole chromosomes or chromosome fragments, as well as by transfer of single genes into tumor cells, has been used as one such test. While a positive result may be meaningful, the test suffers from the frequent rapid elimination of the chromosome regions of interest during tumor growth. Our approach is based on this elimination. We have found using comparative chromosome FISH painting, reverse painting, and PCR marker analysis of microcell hybrids (MCHs) that certain chromosome regions are preferentially eliminated during tumor growth of mouse A9-human microcell hybrid (MCH) cells that originally carried an intact human chr.3. (Elimination test) [18]. We have identified a common eliminated region (CER). The preferential elimination of the CER, during tumor growth in SCID mice is consistent with the possibility that it may harbor (a) tumor suppressor gene(s) [18,20].

We have also found preferential retention of a 3q fragment in parallel with the elimination of 3p. In addition to CER on 3p, we have defined a common retained region (CRR) on 3q(27-q ter) [19]. This suggests that at least one but probably several genes are located in the CER and CRR, respectively, that provide the cell with a negative and positive growth advantage. We suggest that the microcell hybrid system may be suitable, in combination with *in vivo* SCID mouse tumor passages, for the further demarcation and ultimate identification of single genes responsible for both effects.

Detection of aneuploidy by FISH

Multicolor FISH analyses have shown striking heterogeneity in chromosome frequency in interphase cells from solid tumors, both within a given tumor and among tumors of different patients. The ability to associate tumor aggressiveness with specific chromosomal aneuploidy should improve tumor prognosis beyond that possible from measurements of total nuclear DNA content alone.

Detection of structural chromosome abnormalities by FISH

FISH also simplifies the detection of structural chromosomal abnormalities.

For example, we have used chromosome specific painting probe to identify and characterize non-random chromosomal translocations in 2 different B-cell derived tumors: mouse plasmacytomas (MPC) and Burkitt lymphomas (BL). We have showed that identical genetic loci (c-*myc*, Ig) are juxtaposed via chromosomal translocation. We have found in a human B-CLL that the loci (BCL2, IgL) are juxtaposed via

chromosomal translocation t(18;22). While translocations are most readily detected in metaphase cells, they can also be detected in interphase by the disruption of chromosomal domains in interphase after chromosome painting or by the proximity of probes flanking disease-specific breakpoints.

The absence of a fluorescent signal can be diagnostic for deletions (loss of chromosomal regions). Deletions may be very small and thus are undetectable under the microscope. If a sufficiently large probe for the deleted region is available, it is possible to prove a deletion by the fact that after FISH there is only one signal (instead of two signals) on the homologous chromosome.

Somatic cell hybrids containing few human chromosome types on a rodent background are invaluable resources for mapping genes to chromosomes or producing chromosome-specific DNA sequence libraries. However, because human chromosomes can be rapidly lost or rearranged during culture, regular cytogenetic characterization of hybrids is necessary, and is done conveniently with FISH. Human chromosomes (intact, present as fragments or translocated onto rodent chromosomes) are readily detected after hybridization with human genomic DNA [18].

Alternatively, DNA from the hybrid, directly isolated or amplified by PCR, can be used as a probe on human metaphase spreads to highlight the human chromosomal subregions present in the hybrid [20]. This technique is very convenient, but centromeric sequences (through suppression or lack of repetitive sequences) or small chromosomal rearrangements may go undetected.

Gene amplification

Amplified chromosomal regions often manifest as homogeneously staining regions (HSRs) or small acentric (centromere-lacking) chromosomes known as double minute (DM) chromosomes. Gene amplification i.e. the generation of multiple gene copies, in DMs or HSRs, is leading to overexpression of cellular oncogenes. Gene amplification is common in carcinomas and is often associated with advanced malignancy. The spatial resolution and sensitivity of FISH allow localization of parental and amplified genes in solid tumors. For example, we have showed amplification of both c-myc and the linked *pvt*-l locus in a murine sarcoma cell line (SEWA).

Gene mapping

Chromosome maps can be established showing the linear order of DNA fragments on the chromosomes. Such maps are instrumental for identifying probes for further investigations of alterations in the genetic material by means of molecular biology. Regional FISH chromosome maps are an important tool within the project to

generate a complete map of all DNA fragments of the human genome. Our group contributes to this global goal by generating FISH maps of selected regions of the human genome (chromosome 3p21). High resolution mapping of the target chromosome region is also essential for the positional cloning of tumor suppressor genes. For this purpose, we have isolated chr. 3p specific Not-linking clones, cosmids and PACs (bacteriophage PI based artificial chromosome) and mapped and ordered them on chromosome 3 by two color FISH [21–23).

The microcell hybrid panel in combination with FISH permitted the localization of DNA probes to different bands along chromosome 3 [21]. We established a rapid method for identification and mapping of human chromosomes in mouse-human microcell hybrids using the mouse euchromatin specific chromosome painting probe and single copy human chromosome 3 specific probes in two color FISH [24].

High resolution FISH mapping can be achieved by hybridizing probes to artificially extended chromatin or DNA-fibers

High resolution FISH can be conducted using procedures which cause the DNA of chromosomes on a microscope slide to be extended prior to hybridization (extended chromatin fiber FISH) [23]. Such preparations permit extremely high mapping resolutions: from over 700 kb to under 5 kb.

Genome screening

A variety of techniques are now available for genome-wide screening of alteration in copy number, structure and expression of genes and DNA sequences. These include molecular cytogenetic techniques (such as comparative genomic hybridization, spectral karyotyping, and multicolor FISH).

Multiplex FISH (M-FISH)

This approach reported by Speicher et al. [25] uses digital images acquired separately for each of five different fluorophores using a CCD (charge coupled device) camera. The images are analyzed by a software package, which generates a composite image in which each chromosome is given a different pseudocolor depending on the fluorophore composition.

Spectral karyotyping (SKY)

Schrock et al. [26] reported this approach, in which CCD imaging is combined with Fourier spectroscopy. The spectrum of fluorescent wavelengths for each pixel

(picture element) is assessed using an interferometer and a dedicated computer program assigns a specific pseudocolor depending on the particular fluorescence spectrum identified. Chromosome painting has found increasing applications in defining *de novo* rearrangements and marker chromosomes in clinical and cancer cytogenetics. It is particularly helpful in cancer cytogenetics for two reasons. Chromosome preparations from tumors are often of poor quality, but information can often be obtained with the aid of chromosome painting. Additionally, complex chromosome rearrangements are particularly frequent in tumor samples and chromosome paints can be used in combination with standard probes to help recognize particular chromosome segments.

Comparative genomic hybridization (CGH)

One of the most significant new strategies in the analysis of chromosomes in tumors is one, which requires no culture or metaphase preparation from the tumor material and no prior knowledge of the chromosome constitution. Comparative genomic hybridization (CGH) provides a novel way of determining differences between test (tumor) and reference (normal) DNA [27]. The fluorescent signals are quantitated using a digital image analysis system, with a software program to calculate the ratio of red to green fluorescence along the length of each chromosome. Some limitations and pitfalls must be considered, however. CGH cannot detect balanced chromosome rearrangements, no information is available on the way gains and losses are arranged within marker chromosomes and the presence of normal tissues within tumor tissue is a potential problem. It is important to carry out normal/normal hybridization controls for each CGH experiment to determine average fluorescence ratios for the normal chromosomes, as considerable overlap is found between normal and test genomes [28]. Quantitation of fluorescence values may be more reliable using directly fluorochrome labeled probes, rather than immunocytochemical detection. CGH appears to be more sensitive for the detection of amplifications than for deletions, although the limits of sensitivity have yet to be determined. The problems of heterogeneity in tumor samples may be overcome by the use of DOP-PCR amplification of selected areas of tumor tissue followed by CGH analysis. It has been demonstrated recently that this approach is feasible on archival solid tumor samples [29]. A recent modification of reverse painting uses whole tumor genomic DNA as probe [30], which like CGH, results in an increase in fluorescence where there is an over-representation of sequences, and a decrease where there is deletion.

Viral detection and expression

FISH has considerable implications for virologic analysis at the single-cell level. We have recently used this approach to detect Epstein-Barr virus (EBV) genomes in EBV-carrying Burkitt lymphoma (BL) cells. We have also showed that expression of viral genes can be monitored in single cells as well using FISH [31].

Summary

In summary, we have demonstrated here the various ways by which *in situ* hybridization is being applied to establish the genetic composition and organization of complex genomes and how probe sets derived from such studies are being increasingly applied to address important questions in cancer research.

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