

REVIEW ARTICLE

Interphase cytogenetics using fluorescence *in situ* hybridization: an overview of its application to diffuse and solid tissue

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ABSTRACT

Interphase cytogenetics, utilizing fluorescence *in situ* hybridization (FISH) techniques, has been successfully applied to diffuse and solid tissue specimens. Most studies have been performed on isolated cells, such as blood or bone marrow cells; a few have been performed on cells from body fluids, such as amniotic fluid, urine, sperm, and sputum. Mechanically or chemically disaggregated cells from solid tissues have also been used as single cell suspensions for FISH. Additionally, intact organized tissue samples represented by touch preparations or thin tissue sections have been used, especially in cancer studies. Advantages and pitfalls of application of FISH methodology to each type of specimen and some significant biological findings achieved are illustrated in this overview.

INTRODUCTION

The recent development of molecular cytogenetics techniques, such as fluorescent *in situ* hybridization (FISH), has had a major impact on studies aiming to detect and characterize the genetic changes involved in constitutional diseases and in hematopoietic and solid tumors (Poddighe *et al.*, 1992; Cohen *et al.*, 1993; Le Beau, 1993). FISH methodology allows the detection of specific targets not only in metaphase spreads, but also in interphase nuclei, providing a powerful tool for a rapid and sensitive detection of chromosome abnormalities. Interphase cytogenetics utilizing FISH circumvents the need for preparations with dividing cells, overcomes the need

for selective cell growth and allows for genomic screening of different tissues that are otherwise not amenable to routine cytogenetic investigation. In addition, FISH is ideally suited for analysis of single cells, and can greatly contribute to insights into the genetic heterogeneity of biological samples.

Fluorescence *in situ* hybridization: principles and methods

The principle of FISH methodology is based on the ability of single-stranded DNA to anneal to complementary DNA. Briefly, a large number of copies of a particular nucleic acid segment (DNA or RNA) are directly labeled with fluorochromes (fluorescein isothiocyanate - FITC, Texas Red isothiocyanate - TRITC, rhodamine, spectrumOrange, spectrumGreen) or indirectly labeled with haptens (biotin, digoxigenin). The labeled segments are denatured and used as probes to recognize and hybridize to denatured homologous

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sequences in nuclear DNA affixed to microscope slides. After hybridization is allowed to occur under appropriate conditions, unbound or non-specifically bound probes are washed away.

Visualization of the hybridized probes can be accomplished in several ways. Directly labeled probes are identified with a fluorescence microscope, and the number and location of the hybridization signals represent the target sequences in the chromosomal DNA that are complementary to the probe. The fluorescence signal for indirectly labeled probes is detected by immunochemical reactions. Biotinylated probes are usually developed by one or multiple layers of avidin/anti-avidin antibody, coupled with fluorescence tags such as FITC, TRITC or 7-amino-4-methylcoumarin-3-acetic acid (AMCA). The fluorescence signal for digoxigenin-labeled probes is chemically detected by one or multiple layers of antibodies conjugated to FITC or rhodamine. To enable the visualization of the chromosomes and nuclei, the slides are counterstained with DNA-binding fluorochromes, including propidium iodide (PI) or 4,6-diamidino-2-phenylindole (DAPI). Enzymes such as horseradish peroxidase and alkaline phosphatase, or metallic compounds such as colloidal gold or ferritin, may also be used for visualization of the signals in non-isotopic, non-fluorescence assays (Adinolfi, 1992).

Several classes of DNA probes are currently available for FISH, each geared to a specific type of analysis. The most extensively used so far is a large variety of repetitive satellite DNA sequences recognizing specific chromosome structures such as centromeres and heterochromatic or telomeric regions. These repetitive sequences vary in composition so that chromosome-specific probes have been developed for almost all human chromosomes. Repetitive probes produce very bright hybridization signals and have been applied to detect gains and losses of whole chromosomes or chromosome regions. Locus-specific or unique sequence probes amplified in microbiological systems (YACs, cosmids or other vectors) or by polymerase chain reaction (PCR) techniques have been used for identification of small, defined genomic regions. The hybridization signals generated by unique sequence probes are usually smaller than the signals generated by the repetitive probes, but they are discrete and sharp. Partial chromosome gain or loss has mainly been detected using these types of probes. Another useful class of probe is designed as a pool of unique sequence clones homologous to DNA sequences, spanning the length of a chromosome or a target region. These pools are used for "painting" a whole chromosome or a chromosome region and are useful for char-

acterization of complex chromosome rearrangements. Repetitive and unique sequence probes are appropriate for interphase cytogenetics, while the use of the painting probes for interphase analysis is somehow limited by lack of a discrete signal.

Protocols for multiple probe hybridization, which allow the simultaneous delineation of several target sequences in different colors, are currently increasing the potential application of FISH. For instance, Ried *et al.* (1992) reported the simultaneous identification of seven target sequences by using three reporter molecules (biotin, digoxigenin, dinitrophenol), detected by three fluorochromes (FITC, rhodamine, Ultralite). In this study, three of the probes were disclosed by the three fluorochromes, and the other four probes were detected by mixtures of two (1:1) or of the three (1:1:1) fluorochromes.

Diffuse and solid tissue as target for FISH

FISH has been applied to a large variety of biological specimens, including cell suspensions derived from diffuse tissue, disaggregated solid tissue and external body fluids, as well as solid tissue imprints onto glass slides, and thin tissue sections. An illustration of these applications focusing on advantages and pitfalls, and some examples of interesting findings using FISH methodology are provided in this overview.

Single cell suspensions provide excellent material for FISH analyses, and are increasingly explored substrates for both constitutional and cancer cytogenetics. In single nucleus preparations, the whole nucleus is observed under the microscope. The hybridization signals are usually in the same plain and there is a low background interference. However, single cell suspensions destroy any information on the relative positioning of different cells with respect to each other. On the other hand, touch preparations and paraffin-embedded tissue sections maintain the relative positioning of the cells in the sample, but may exhibit partially sliced nuclei. Searching for numerical abnormalities in these preparations can be problematic since partially sliced nuclei may give a false picture of the findings. FISH results are expressed by the copy number of the markers detected, and loss of signal in partial nuclei is inseparable from loss of signal in intact nuclei. Genomic or chromosomal deletional events may be enhanced and gains may be concealed when FISH analysis is performed on this type of substrate. In addition, overlapping cells in touch preparations and tissue sections make the analysis less rapid and less accurate.

Another important factor to be considered in the comparison between single cell suspension and touch preparation or tissue section analyses is related to the sensitivity of the test. In cases in which only a small fraction of cells presents the genetic abnormality, which may occur in early or focal clones, preparations that keep the morphological architecture of the tissue are more likely to detect these clones, otherwise lost by tissue disaggregation.

Analysis in single-cell suspensions: uncultured cells and cell smears

Interphase cytogenetic analysis in single cell suspensions has been applied to constitutive cytogenetic and oncological approaches. Probably the most frequent application for FISH in clinical cancer is in the diagnosis and follow-up of leukemia in fresh and aged bone marrow smears. Hammond *et al.* (1994) reportedly used bone marrow smears of children with acute lymphoblastic leukemia (ALL), either Romanowsky stained, or frozen, unstained and preserved for up to 20 years, for FISH tests with centromeric probes specific for chromosomes X, 6, and 16. The hybridization efficiency ranged from 76 to 100%, with the older specimens not performing as well as the more recent samples. A trisomic clone was defined in an ALL bone marrow smear when the percentage of nuclei displaying three signals was greater than the mean percentage plus two times the standard deviation of cells in the remission samples. Using this criterion, all cases of ALL smears previously known to contain extra chromosomes identified by conventional cytogenetics were clearly detected by FISH. For the series of children with ALL in remission, the hybridization efficiency ranged from 51 to 99%, with an average of 77%. According to these authors, the pyknotic nuclei of late erythroblasts might show reduced hybridization efficiency, thus contributing to the number of negative nuclei in remission samples.

The efficacy of FISH tests for opposite sex bone marrow transplantation studies using centromere enumeration probes for chromosomes X and Y was well demonstrated in interphase cells by Dewald *et al.* (1993a). Male donors exhibited X and Y signals in 99% of cells, while female donors exhibited two X-chromosome centromeric signals in 97% of the cells. In transplanted specimens, XX donor cells were detected in each of the 11 male patient samples and XY donor cells were seen in each of the 11 female patient samples, thus providing a sensitivity of 100%. By comparison, the sensitivity for conventional cytogenetics was 77%. These findings suggested that FISH is particularly

useful for analyses in hypoplastic bone marrow specimens, in which only a few cells are available for analysis. Moreover, the retention of cell morphology following application of FISH protocols permits the identification of hematopoietic cell types and distinction between blast and mature cells.

Another interesting example of the application of FISH to bone marrow smears of cancer patients was presented by Taylor *et al.* (1994), in which *N-myc* amplification, ploidy and chromosome 1p deletions were investigated in neuroblastoma patients. Bone marrow specimens showed infiltration with neuroblastoma cells, which appeared singly or in clumps in the smears. Tumor cells were identified by Giemsa staining prior to FISH, without altering the hybridization efficiency. The assessment of ploidy and the detection of chromosome 1p aberrations, using FISH, were highly correlated with the karyotyping findings in the three patients in which classical cytogenetics results were available. Similarly, the absence of *N-myc* amplification by FISH correlated positively with the Southern blot results.

Since bone marrow cells from patients undergoing therapy frequently lack a sufficient number of mitotic cells for karyotype analysis, interphase FISH is potentially a valuable tool for monitoring therapy response. This application was illustrated by the detection of cells with chromosome 7 monosomy in patients with hematological neoplasias, following chemotherapy with several agents (Zhao *et al.*, 1993), as well as the detection of cells with t(15;17) in acute promyelocytic leukemia patients, after treatment with tretinoin (Warrell Jr. *et al.*, 1991). FISH methodology also aggregates the best features for detection of minimal residual disease. The reliability of FISH to estimate the proportion of Philadelphia chromosome-positive cells in patients with chronic myeloid leukemia before and/or after treatment using the bcr-abl probe was elegantly demonstrated by Dewald *et al.* (1993b).

In the clinical approach, interphase cytogenetics with DNA probes can be illustrated by the identification of specific aneuploidies in direct cytotrophoblastic preparations of chorionic villus samples (CVS), and in uncultured amniocytes. This alternative path to conventional cytogenetics studies has been successful for determining the copy number of autosomes and sex chromosomes in prenatal diagnosis. Harrison *et al.* (1993) and Rao *et al.* (1993) demonstrated that analysis of a large number of CVS cells can be rapidly performed. FISH procedures permit not only identification of chromosome aneuploidies, but also rule out the presence of contaminating maternal cells in the villus samples from a male pregnancy. In addition, interphase cytogenetic analysis was considered an

excellent method for identifying confined placental mosaicism following prenatal diagnosis.

Aneuploidy detection by FISH in uncultured amniocytes was reported by Klinger *et al.* (1992) in a study performed on 526 amniotic fluid samples. These authors constructed cosmid-contig probes derived from specific subregions of human chromosomes 13, 18, 21, X and Y. The samples were analyzed in a blind fashion and the results obtained on interphase nuclei were compared with those from cytogenetic analysis. Samples in which less than 23% of the nuclei displayed three hybridization signals were predicted to be disomic for a given chromosome, while samples in which more than 42% of the nuclei exhibited three signals were predicted to be trisomic. The hybridization patterns that generated 23 to 42% three-signal nuclei were considered as uninformative. By these criteria, aneuploidies for all five chromosomes tested were easily identified.

Another interesting example of FISH application was provided by Ward *et al.* (1993) with uncultured amniocytes. DNA probes for chromosomes 13, 18, 21, X, and Y were used to determine cell ploidy by analysis of signal number in 4329 samples. Samples were considered as diploid when all autosome probes generated two hybridization signals and a normal sex chromosome pattern was observed in at least 80% of nuclei. Samples were identified as trisomic or monosomic when at least 70% of nuclei displayed the same abnormal hybridization pattern for a specific probe. When all probes demonstrated a trisomic pattern, a triploid chromosome constitution was inferred. Ninety percent of the specimens met these criteria and the remaining specimens were reported as uninformative.

Cacheux *et al.* (1994) demonstrated that FISH with X, Y, and 18 alpha satellite DNA probes could accurately detect aneuploidies involving these chromosomes, and could be used in prenatal clinical laboratories. In contrast, the 13/21 alpha satellite DNA probe hybridizing both to chromosomes 13 and 21 was considered unreliable for prenatal diagnosis in uncultured amniocytes, because hybridization domains with this probe varied between one and six spots, both in normal and trisomic samples. Undetectable centromeric domains of chromosome 21 have been previously reported by Verma and Luke (1992) in two siblings born consecutively with Down syndrome exhibiting nuclei with only four spots instead of five. The father, who was cytogenetically normal and should have cells with four spots, showed only three spots. Similarly, Mizunoe and Young (1992) have reported occurrence of only three hybridization spots indicating

monosomy of chromosome 21 or chromosome 13 in CVS of a cytogenetically normal female.

A technical problem sometimes associated with FISH analysis in amniocytes, as well as in other cell types, is related to the degree of chromatin condensation. Cacheux *et al.* (1994) described nuclei from uncultured amniocytes as smaller, more condensed, and presenting less intense and more patchy hybridization signals than nuclei from cultured amniocytes. Moreover, about 50% of the nuclei were degenerated or covered by residual cytoplasm, and most of them exhibited no hybridization signal. Washing in glacial acetic acid and pepsin digestion were shown to largely improve the intensity of the hybridization domain under such adverse conditions (Varella-Garcia *et al.*, 1995).

FISH was demonstrated as a useful technique for the diagnosis of sex and ploidy assessment in nucleated erythrocytes from newborn cord blood, as a non-invasive prenatal diagnostic test. Multicolor FISH with alpha satellite probes for chromosomes X and Y, and cosmid probes from the Down's syndrome region in cells from normal male cord blood samples showed that greater than 95% of the cells exhibited the expected number of signals (Rao *et al.*, 1994). However, nucleated red blood cells are extremely fragile and appear not to withstand the harsh retrieval treatment, making analysis somewhat difficult.

An additional example of biological material tested by interphase cytogenetic for autosomal trisomy or gender determination in newborn infants is the buccal smear. Centromeric probes for chromosomes 8, 18, X, and Y were used by Harris *et al.* (1994). The overall probe efficiency for detecting expected chromosome number in these interphase cells was about 70%, which was significantly lower than the 95% of efficiency routinely obtained with interphase nuclei from peripheral blood harvests in the same laboratory. Loss of signal was attributed to the presence of karyopiknotic intermediate epithelial cells, that may take up counterstain, even though the DNA is degraded beyond the point of reliability, resulting in high false monosomy rates. Thus, although FISH can be applied to buccal smears for rapid diagnosis of numerical chromosome aberrations in newborns, probed buccal smear specimens may not be accurate at diagnosing mosaicism.

In situations in which the available number of cells is very low, such as in fine needle aspirates, interphase cytogenetics by FISH proved to be a powerful tool for determining numerical chromosomal abnormalities. Fine needle aspirates derived from patients with various types of neoplasm were evaluated by Cajulis and Frias-Hidvegi (1993). Fresh specimens were

smear onto slides in a monolayer fashion, incubated in collagenase to digest associated cell stroma and fixed in formaldehyde. Hybridization was performed with DNA probes specific for chromosomes 8 and 12, and numerical aberrations were detected in 85% of the malignant samples while a normal number of chromosomes was found in the benign samples. Technical difficulties in detecting and counting hybridization domains were observed in areas with overlapping cells, thus the cell density needs to be controlled. The presence of abundant inflammatory cells (polymorphonuclear leukocytes and lymphocytes) covering the nuclei of the cells of interest is another technical artifact to be considered in the FISH protocol, when using smears derived from fine needle aspirates.

Analysis of isolated cells from external body fluids: urine, sperm, and sputum

Utilization of cells from external body fluids for the detection of constitutional or acquired genetic anomalies has several advantages. Sample collection is non-invasive, so there is little discomfort to the patient. Samples are readily available and tracking of patients' progress through periodic testing is relatively easy and cost-effective. Several sources of material such as urine, sperm, and sputum are currently under study using FISH techniques. Most of these studies aim at early diagnosis of cancer or early detection of genetic events in cancer.

The FISH technique can be useful for the diagnosis and follow-up of bladder cancer (Meloni *et al.*, 1993). Tumor tissue and cells pelleted from urine samples from patients, and cells from urine samples from controls were hybridized with centromeric probes for chromosomes 7, 8, 9, 10, 11, X, and Y. A very high correspondence of FISH results between urine and tumor tissue cells was observed, suggesting that interphase cytogenetics on urine cells is an efficient tool in the detection and management of bladder cancer.

Sputum samples have been tested to monitor malignant and pre-malignant stages of lung cancer, using molecular markers from chromosomes 3 and 7 (Vartanian *et al.*, 1995). Specimens were mechanically disaggregated into mononuclear suspensions, washed in acetic acid, digested with RNase A and pepsin, fixed in formaldehyde, and submitted to a dual color FISH protocol. Polyploidy, suspected by the increased number of copies of positive domains for chromosomes 3 and 7 in the same cell, was detected in sputum from patients with adenocarcinoma in this study.

FISH has also been applied to detect the proportion of X- and Y-chromosome bearing sperm,

and to estimate the incidence of diploidy and disomy for the sex chromosomes in human sperm of normal males (Williams *et al.*, 1993; Robbins *et al.*, 1993; Wyrobek *et al.*, 1994). Chromatin decondensation in sperm smears was accomplished by consecutive incubations in dithiothreitol and lithium diiodosalicylate. This procedure yields bright and well-delineated fluorescent signals suitable for determining the frequencies of chromosome X- and Y-carrying cells. However, reliable hybridization was not obtained in cells smaller than 5 μm due to insufficiently expanded chromatin and cells larger than 10 μm showed diffuse and poorly delineated hybridization domains, limiting the number of scorable cells.

An additional interesting example of interphase cytogenetic application in body fluids was a search for chromosomal abnormalities in human sperm after chemotherapy, by multicolor FISH (Martin *et al.*, 1995). Using specific probes for chromosomes 1, 12, X, and Y, these authors ruled out any increase in the frequency of numerical chromosomal abnormalities in a patient, compared to control donors.

Analysis of isolated nuclei from solid tissues: fresh, fresh-frozen, and formalin-fixed, paraffin-embedded specimens

Fresh or preserved solid tissue specimens are usually transformed into nuclear suspensions for FISH analysis. Mechanical and chemical procedures are available for the disaggregation of solid tissues, which can also be achieved by microdissection of cells from sections fixed to microscope slides. Disaggregation techniques have greatly increased FISH applications, particularly in cancer cells. Microdissection methodology permits the selection of particular cells carrying specific markers. Furthermore, use of isolated nuclei avoids signal loss due to cell nuclei being cut partially. Analysis of isolated nuclei is useful in cases where a low level of mosaicism for monosomy is suspected.

Optimization of single and double-target FISH protocols using probes for chromosomes 1 and 18 in single cell suspensions from fresh bladder tumor tissue was first reported by Hopman *et al.* (1988, 1989). These authors demonstrated that cell suspensions can be stored for more than three years without noticeable differences in FISH results. They also found that mechanical disaggregation occasionally leaves some cytoplasm around the nucleus, which leads to a high auto-fluorescent background and strongly reduces the hybridization reaction. Washing off the slides with 70% acetic acid prior to hybridization diminished these problems, by removing the residual cytoplasm with no

changes in the cell morphology. Furthermore, acetic acid flattens the cells onto the slide, which means that fluorescent signals are more likely to be in the same plane and the need for multiple focusing is decreased.

Isolated nuclei from fresh-frozen tissue have been an increasingly explored substrate for FISH studies in cancer research. This type of preparation was used, for instance, to investigate structural aberrations of chromosome 17 in 18 malignant gliomas by Kwak *et al.* (1995). The frozen specimens were defrosted, minced, filtered, submitted to hypotonic treatment, and fixed in methanol:acetic acid. Hybridization was performed with DNA probes specific for centromeric, telomeric, and 17p13.1 (TP53) sequences of chromosome 17. Short arm deletions of 17p including the TP53 gene were more often detected in anaplastic astrocytomas than in glioblastomas. These results supported the hypothesis that TP53 deletions are associated with the early events in glial tumorigenesis and that some glioblastomas are independent from tumor progression through low-grade gliomas.

An example of FISH in isolated nuclei from paraffin-embedded tissue is illustrated by Schofield and Fletcher (1992). In this study, trisomy 12 was detected in pediatric granulosa-stromal cell tumors with a centromeric probe. Briefly, one to three 60- μ m tissue sections were cut and mechanically disaggregated. Deparaffinization in xylene was followed by digestion with pepsin. The fragments were vigorously drawn up and down in a syringe and filtered through a nylon mesh before being pipetted onto glass slides and submitted to hybridization. Although a variable signal intensity was observed, the hybridization domains were easily counted, even in specimens preserved for 25 years.

This technique of Schofield and Fletcher (1992) was slightly modified by Lee *et al.* (1993), who achieved a higher sensitivity for alpha-centromeric probes and also analyzed whole chromosome painting probes in nuclei from synovial sarcoma cells. Signals for alpha-centromeric probes for chromosomes X and 18 were detected in about 95% of cells. However, with painting probes, frequency of disomic nuclei was only about 85%, which can be explained by the lack of a discrete signal from the painting probes. The authors could identify interphase cells carrying a translocation involving chromosomes X and 18, by the presence of two alpha-centromeric signals and three painting signals. A high correlation between classical cytogenetic findings and FISH results was demonstrated in this study.

Nuclei isolated from different types of tissue have exhibited variable responses in FISH efficiency.

The addition of a hot glycerol incubation to the sample preparation protocol has improved probe penetration in disaggregated breast tumor cells, either from frozen or from formalin-fixed, paraffin-embedded tissue (Hyytinen *et al.*, 1994). Without previous treatment, no scorable hybridization signal was obtained in the formalin-fixed tumor samples while the hybridization efficiency on normal paraffin-embedded lymph nodes in the same experiment ranged from 0 to 50%, when using centromeric probes for chromosomes 4, 8, 10, and 16. Treatment of the samples for 3 min in a hot 50% glycerol/0.1x SSC solution raised the mean percentage of lymph node nuclei with two signals to 91% and of the tumor samples to about 90%. Trisomy or tetrasomy for at least one of these chromosomes was observed in several paraffin-embedded samples, as well as in fresh frozen specimens.

With the use of the hot glycerol incubation in the pre-treatment of the cells, FISH was considered more sensitive than flow cytometry for aneuploidy detection by Visakorpi *et al.* (1994). They investigated the pattern of chromosomal aberrations in prostate cancer and in benign prostatic hyperplasia, using pericentromeric probes specific for 10 human chromosomes. Tumors were identified as trisomic or tetrasomic if more than 10% of nuclei showed three or four signals, and monosomic if more than 20% nuclei showed only one signal. All of the benign hyperplasia specimens exhibited a diploid DNA content by DNA flow cytometry and showed only disomic cells with the chromosome probes. In prostate cancer specimens, 35% of samples exhibited abnormal DNA content by flow cytometry, whereas 74% had abnormalities detected by FISH. The most frequent aberrations, mostly chromosome gains, affected chromosomes 8, X, and 7, respectively. One interesting observation was that 94% of all the aneuploid cases detected by flow cytometry and/or FISH would have been detected by FISH, targeting only these three chromosomes.

Analysis in undissociated tissue:

a) Formalin-fixed, paraffin-embedded sections

The preservation of histological context in paraffin sections allows for the analysis of the topographical distribution of cytogenetically abnormal cells. Moreover, no selection of cells occurs as a result of the culture or cell disaggregation procedures (Poddighe *et al.*, 1992). However, there are only a few reports utilizing FISH on tissue sections. Examples include interphase cytogenetic analyses in formalin-fixed sections of routinely processed hydatidiform moles and

in hydropic abortions (Van de Kaa *et al.*, 1991), archival tissues from infants with multiple congenital malformations (Drut *et al.*, 1992) and solid tumors, such as breast, bladder, prostate and brain tumors (Devilee *et al.*, 1988; Hopman *et al.*, 1991; Poddighe *et al.*, 1992; Henke *et al.*, 1993; Paulus *et al.*, 1994).

FISH analysis on tissue sections from 11 prostate cancer specimens was developed by Henke *et al.* (1993), using centromeric probes for chromosomes 7, 10, 17, X, and Y. Numerical aberrations involving at least one of those chromosomes were found in five cases of advanced stage and large volume high-grade tumors, while the specimens with normal chromosome numbers came from smaller and prostate-restricted tumors. Chromosome number variations were observed in different tumoral areas in four cases, and could be related to changes in histological differentiation. However, a similar study conducted by Alers *et al.* (1995) showed genetic heterogeneity, i.e., subsets of tumor cells carrying a chromosomal abnormality, even in low-grade, early-stage prostate adenocarcinomas.

Interesting results were reported by Paulus *et al.* (1994) in gliomas. The authors described a large intra-tumoral cytogenetic heterogeneity with formation of monoclonal cell clusters in glioblastomas and gliosarcomas. In this study, the finding that more than 45% of the nuclei had only one hybridization domain was interpreted as an indication of tumor monosomy. More than 7.5% of the nuclei with three signals were regarded as indicating a tumor subpopulation with trisomy. Losses of chromosomes 10, 17, and X were observed in tumor cells, whereas the non-neoplastic cells were normal. In one gliosarcoma, gain of the X chromosome was observed in the sarcoma portion, whereas the glioma portion exhibited monosomy 10. These results suggest different histogenetic pathways for the sarcoma and glioma elements in this gliosarcoma.

b) Touch preparations

Undissociated tissue has been more rarely used for FISH than cell suspensions, and analysis of touch preparations is more infrequent than in tissue sections. Touch preparations can be obtained from fresh tissue samples prior to routine formalin-fixation procedures, or from tissue previously frozen at -70°C (Matsumura *et al.*, 1992; Persons *et al.*, 1993a,b; Jones *et al.*, 1994; Taylor *et al.*, 1994). Imprints can be made directly onto coated slides using a dry, blood-free, newly cut surface of the biopsy material, thus allowing loosely adherent tumor cells to stick to the slide while maintaining their organizational context. Slides can be fixed either in cold

methanol, 95% ethanol or 3:1 methanol:acetic acid, air dried, baked at 65°C for about 4 h and stored at -20°C until *in situ* hybridization is performed. A great advantage of this type of preparation is that very little material is required, compared with tissue culture, Southern blotting or fluorescence-activated cell sorting (Taylor *et al.*, 1994). Furthermore, the use of touch preparations made directly from the tumor specimens ensures analysis of abnormalities that are present *in vivo* and not those selected in tissue cultures.

Touch preparations of 25 primary ovarian carcinomas were examined with two color combinations of centromeric probes for chromosomes X, 8, 12, and 17, and an HER-2/*neu* oncogene probe by Persons *et al.* (1993a). To reduce sampling error because of the potential heterogeneity of chromosomal abnormalities within the touch preparations, several areas were evaluated independently by two observers. The modal centromere copy numbers were defined as comprising at least 20% of the 500 cells evaluated, and used as a reference for further analysis. Losses of chromosomes 17 and X, and gains of chromosomes 8 and 12, were the most common findings in this series of samples. In addition, amplification of the HER-2/*neu* gene was detected in two of the 25 tumors analyzed.

Variable proportions of normal and tumor cells may be observed in different areas within a touch preparation, reflecting the different infiltrating patterns of the tumor within normal tissues. However, touch preparations may be composed primarily of tumor cells because these cells are less cohesive in neoplastic tissue (Jones *et al.*, 1994). An evaluation of touch preparations from 20 prostate tumor specimens with pericentromeric FISH probes specific for chromosomes 7 and 17 was reported by Jones *et al.* (1994). The alpha satellite X chromosome probe was used for assessment of the ploidy of each cell and of hybridization efficiency. Cells without chromosome X hybridization signal were assumed to be the result of poor hybridization, and were not scored. These authors established, from control studies, significance levels of 15% or greater for chromosome loss and 5% or greater for chromosome gain. In touch preparations, chromosome 17 was lost in 12 specimens and chromosome 7 was gained in two specimens. Two specimens were presumed to be tetraploid or pseudotetraploid, based on the observation of two X chromosome signals and three or more autosome signals. No interpretable FISH data for chromosome 17 were obtained in two cases and two other samples gave no satisfactory results for chromosome 7.

In parallel with FISH analyses on bone marrow smears from patients with neuroblastoma as previously described, Taylor *et al.* (1994) also used touch

preparations for the visualization of relevant genetic changes. The quality of the *in situ* hybridization was considered higher in the tumor imprints than in the marrow smears, as there was less background fluorescence and fewer areas of non-tumor cells. Similarly, as observed in bone marrow smears, the FISH results correlated well with the karyotyping and Southern blotting data.

Most of the studies involving touch preparations were performed with tumor samples, but FISH analysis is also possible in touch preparations from spontaneous abortions, stillbirths and molar pregnancy specimens. Fresh specimens that cannot be cytogenetically studied due to a lack of cell growth or to extreme bacterial and fungal contamination may be smeared or touched to slides, allowed to air dry and processed for FISH, usually with high efficiency, as reported by Harris *et al.* (1995).

Concluding remarks

Although many technical problems still limit the current application of FISH to non-dividing cells, interphase cytogenetics using this methodology has been an increasingly appropriate approach in addressing important biological questions. Methodological advances in both sample and probe preparations, as well as in the image analysis systems for acquisition and processing of the images and analysis of the hybridization signals, will clearly assist the future use of FISH in diagnostic procedures. As a consequence, extended use of FISH for clinical applications is occurring. Furthermore, a significant increase in the essential insights already brought by FISH into several issues, such as construction of physical maps of chromosomes, analysis of chromosome structure and abnormalities, investigation of the structure, function and evolution of genomes, determination of the spatial and temporal expression of genes, and localization of oncogenes and tumor suppressor genes, is expected shortly.

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RESUMO

Técnicas para marcação de hibridação *in situ* por fluorescência (FISH) têm sido amplamente utilizadas para

análise citogenética em células interfásicas de tecidos sólidos e difusos. Na maioria dos casos, os estudos com FISH têm sido desenvolvidos em células naturalmente isoladas, como as células sanguíneas ou de medula óssea. Mais raramente, a análise citogenética tem sido desenvolvida em fluidos corporais como líquido amniótico, urina, esperma ou escarro. As amostras de tecidos sólidos, na maioria dos casos, são submetidas à desagregação mecânica ou química anteriormente à aplicação de FISH. Contudo, FISH também tem sido aplicada a amostras de tecido com arquitetura conservada, tais como os "imprints" ou cortes histológicos finos, principalmente em estudos com tumores sólidos. Este artigo exemplifica vantagens e limitações da técnica, ilustra o uso de FISH para análise citogenética em células interfásicas de diferentes tipos e destaca alguns resultados interessantes obtidos com a metodologia.

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